

# **GLUCAGON-LIKE PEPTIDE-1 IN NONALCOHOLIC STEATOHEPATITIS**

**By**

**Dr MATTHEW JAMES ARMSTRONG**

**MBChB(Hons) BSc(Hons) MRCP**

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**NIHR Liver Biomedical Research Unit  
& Centre for Liver Research  
School of Immunity and Infection  
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## ABSTRACT

Nonalcoholic fatty liver disease (NAFLD), and in particular its inflammatory component steatohepatitis (NASH), are associated with significant risk of liver/cardiovascular morbidity and death. My findings highlight that NAFLD is now the commonest cause of liver disease in primary care, yet significant numbers with advanced fibrosis remain undetected. Application of simple non-invasive scoring systems could aid with identifying those in greatest need of intervention.

By adopting an integrative physiological approach with functional measures of lipid and carbohydrate flux, I demonstrated that patients with NASH (vs. healthy controls) have marked adipose tissue dysfunction (especially in abdominal subcutaneous adipose tissue), alongside increased hepatic and muscle insulin resistance (IR). Targeting adipose-derived lipotoxicity should be the mainstay of therapy in NASH. Glucagon-like peptide-1 (GLP-1) based therapy (liraglutide) appears to be safe and well tolerated in patients at risk of underlying NAFLD. My prospective randomised-controlled study highlighted that liraglutide reduces metabolic dysfunction, hepatic lipogenesis, hepatic/adipose IR and inflammation in patients with NASH. My *in vitro* studies in human hepatocytes indicate that the anti-steatotic effects are not solely reliant on improvements in weight and/or glycaemic control. Taken together, my findings highlight that GLP-1 based therapies have all the metabolic and clinical attributes to make them a promising therapeutic option in patients with NASH. However, the safety and histological efficacy of such awaits the completion of my 48-week Phase II 'LEAN' trial, which is integral as to whether larger clinical trials are warranted.

## **DEDICATION**

My thesis is dedicated to my beautiful wife Caroline, my long suffering parents Barbara and John, and my big sis Emma. I would not be where I am today without their endless support and devotion.

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## LIST OF ABBREVIATIONS

### A

A1AT - alpha 1 antitrypsin deficiency  
 ACC - acetyl carboxylase  
 AE - adverse event  
 AFP - alpha-feta protein  
 AIH - autoimmune hepatitis  
 ALIOS - American lifestyle-induced obesity syndrome  
 ALP - alkaline phosphatase  
 ALT - alanine aminotransferases  
 AMPK - adenosine monophosphate- activated protein kinase  
 ANCOVA - analysis of covariance  
 APRI - AST/platelet ratio index  
 ARFI - acoustic radiation force impulse  
 AST - aspartate aminotransferases  
 ATP - adenosine triphosphate  
 ATP III - Adult treatment Panel III  
 AUC - area under the curve  
 AUDIT - Alcohol use identification test

### B

BALLETS - Birmingham and Lambeth Liver Evaluation Testing Strategies  
 BD - bi-daily  
 BEC - biliary epithelial cells  
 BMI - body mass index  
 BP - blood pressure  
 BSA - bovine serum albumin

### C

cAMP - cyclic adenosine monophosphate  
 cDNA - complementary deoxyribonucleic acid

CCL - chemokine ligand  
 ChREBP - carbohydrate responsive element-binding protein  
 CKD - chronic kidney disease  
 CK-18 - cytokeratin 18  
 CNS - central nervous system  
 CoA - coenzyme A  
 CPT - carnitine palmitoyl transferase  
 CRCTU - cancer research clinical trials unit  
 CRF - case report form  
 CRP - C-reactive protein  
 CT - computer tomography  
 CVD - cardiovascular disease

### D

DEXA - Dual-energy X-ray absorptiometry  
 DMC - data management committee  
 DMEM - Dulbecco's modified Eagle's medium  
 DNL - *de novo* lipogenesis  
 DPP-4 - dipeptidyl peptidase 4

### E

ECL - enhanced chemiluminescence  
 EGP - endogenous glucose production  
 ELF - Enhanced liver fibrosis  
 EMA - European Medicines Agency  
 EOT - end of treatment  
 ER - extended release

### F

FAS - fatty acid synthase  
 FCS - foetal calf serum  
 FDA - Food and Drug Administration  
 FLD - fatty liver disease

## **G**

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

Gd - glucose disposal

GGT - gamma-glutamyl transferase

GIP - gastric inhibitory polypeptide

GLP-1 - glucagon-like peptide-1

GLP-2 - glucagon-like peptide-2

GLP-1R - glucagon-like peptide-1 receptor

GPCR - G-protein coupled receptor

## **H**

H&E - haematoxylin & eosin

HbA1c - glycated haemoglobin

HBV - viral hepatitis B

HCC - hepatocellular carcinoma

HCV - viral hepatitis C

HDL - high density lipoprotein

HFD - high fat diet

H-MRS - protein magnetic resonance spectroscopy

HOMA-B - homeostatic model of assessment of  $\beta$ -cell activity

HOMA-IR - homeostatic model of assessment of IR

HSEC - human sinusoidal endothelial cell

## **I**

IFG - impaired fasting glucose

IGT - impaired glucose tolerance

IL- - interleukin-

INR - international normalised ratio

IQR - interquartile range

IR - insulin resistance

IRMS - isotope ratio mass spectrometer

IRS - insulin receptor substrate

ITT - intention-to-treat

## **L**

LEAD - Liraglutide Efficacy and Action in Diabetes

LFT- liver function tests

LOCF - last observation carried forward

LSAR - liver:spleen attenuation ratio

## **M**

MCP-1 - monocyte chemo-attractant protein 1

MEN - multi-endocrine neoplasia

MHRA - Medicines and Healthcare Products Regulatory Agency

MRI - magnetic resonance imaging

MTC - medullary thyroid carcinoma

MTT - methylthiazolylphenyl-tetrazolium

MTTP - microsomal triglyceride transfer protein

## **N**

NAFL - nonalcoholic fatty liver

NAFLD - nonalcoholic fatty liver disease

NAS - NAFLD activity score

NASH - non-alcoholic steatohepatitis

NCEP - National Cholesterol Education Program

NEFA - non-esterified fatty acids

NFS - NAFLD Fibrosis Score

NHANES - National Health and Nutrition Examination Survey

NHS - National Health Service

NPV - negative predictive value

## **O**

OAD - oral anti-diabetic drug

OD - once-daily

OR - odds ratio

## **P**

P3NP - procollagen 3 N-terminal propeptide

PBC - primary biliary cirrhosis

PBS - phosphate buffer solution

PCP - primary care practitioner

PDK - phosphoinositide-dependent kinase-1

PIK-3 - phosphatidylinositol 3-kinase

PKA - protein kinase A

PKC - protein kinase C

PPAR - peroxisome proliferator-activated receptors

PPV - positive predictive value

PSC - primary sclerosing cholangitis

## **Q**

QoL - quality of life

## **R**

R&D - Research & Development

RCT - randomised-controlled trial

RNA - ribonucleic acid

RT - reverse transcriptase

## **S**

SAE - serious adverse event

SAT - subcutaneous adipose tissue

SCD-1 - stearoyl CoA desaturase

SmPc - Summary of product characteristics

SRBEP - sterol regulatory element-binding protein

SUSAR - suspected unexpected serious adverse event

## **T**

TIMP-1 - tissue inhibitor of metalloproteinases 1

TNF $\alpha$  - tumour necrosis factor alpha

TSH - thyroid stimulating hormone

TZD - thiazolidinediones

## **U**

UHB - University Hospital Birmingham

UK - United Kingdom

US - United States

USS - ultrasound

## **V**

VAT - visceral adipose tissue

VLDL - very low density lipoprotein

## CHAPTER 1: GENERAL INTRODUCTION

### 1.1 Nonalcoholic fatty liver disease (NAFLD)

Nonalcoholic fatty liver disease (NAFLD) is a clinico-pathological entity that encompasses simple hepatic steatosis, necroinflammation with varying stages of fibrosis known as non-alcoholic steatohepatitis (NASH), and cirrhosis. It is strongly associated with the metabolic syndrome and is the leading cause of chronic liver disease worldwide (Anstee et al., 2013). Compared with the general population of similar age and gender, NAFLD increases the risk of end-stage liver disease, hepatocellular carcinoma (HCC) (El-Serag et al., 2004), as well as liver-related and all-cause mortality (Adams et al., 2005b; Ekstedt et al., 2006; Söderberg et al., 2010; Musso et al., 2011). Subsequently, NAFLD is expected to become the commonest indication for liver transplantation in forthcoming years (Charlton et al., 2011). The cumulative evidence to date also suggests that patients with NAFLD (and specifically NASH) harbour an increased and (*in part*) independent risk of developing cardiovascular disease (CVD), type 2 diabetes, chronic kidney disease (CKD) and extra-hepatic malignancy (Armstrong et al., 2013a). The current management of NAFLD includes lifestyle intervention and aggressive management of CVD risk factors, but there remains no universally accepted pharmaceutical option for NAFLD - and more importantly NASH. Identifying therapies that target not just intrinsic liver pathology, but also the co-existing metabolic dysfunction (i.e. central adiposity, insulin resistance, dyslipidaemia) that collectively drive the progression of NAFLD, is a critical unmet need in public health.

### **1.1.1 Definition of NAFLD**

NAFLD was first described by Ludwig and colleagues in 1980 (Ludwig et al., 1980). It is characterised by the following features: 1) pathological fat accumulation within the liver, which is predominantly macrovesicular (>5% hepatocytes) on histological assessment; and 2) absence of excessive alcohol consumption and other known causes of chronic liver disease. The latter includes viral hepatitis B/C (HBV/HCV), autoimmune diseases (autoimmune hepatitis (AIH), primary sclerosing cholangitis (PSC), primary biliary cirrhosis), genetic diseases (haemochromatosis, Wilsons disease, alpha-1 anti-trypsin (A1AT)) and drug-induced liver disease.

A consensus meeting in 2011 defined the threshold for excessive alcohol consumption as 20g of ethanol per day in women (~14 units/week) and 30g per day in men (~21 units/week) (Sanyal et al., 2011). Prior to this, published studies on NAFLD had been inconsistent with regards to acceptable limits of ethanol and with a lack of gender specificity. It is imperative that an accurate alcohol history is established prior to diagnosing NAFLD, as the two disease entities are practically indistinguishable on imaging or histological assessment (Hübscher, 2006). After exclusion of alcohol intake, other causes of hepatic steatosis should be considered prior to confirming a diagnosis of NAFLD (Table 1-1).

NAFLD is not a single disease entity, but rather a spectrum of diseases that include simple hepatic steatosis, necroinflammation and early fibrosis through to cirrhosis (Brunt et al., 1999; Matteoni et al., 1999). Nonalcoholic fatty liver (NAFL) is the term used to describe the

presence of hepatic steatosis in the absence of hepatocyte ballooning, necroinflammation and fibrosis. Whereas NASH, the more aggressive form of NAFLD, is defined as a single disease entity by the histological presence of >5% macrovesicular steatosis, necroinflammation, hepatocyte ballooning in the presence or absence of fibrosis (Brunt et al., 1999; Kleiner et al., 2005).

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#### **Macrovesicular hepatic steatosis**

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Abetalipoproteinaemia

Lipodystrophy

Medications (e.g. amiodarone, corticosteroids, methotrexate, tamoxifen, oestrogens)

Nutritional causes (prolonged starvation, parenteral feeding)

Other causes of chronic liver disease (genotype 3 HCV, excessive alcohol & Wilson's disease)

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#### **Microvesicular hepatic steatosis**

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Acute fatty liver of pregnancy

HELLP syndrome

Inborn errors of metabolism (e.g. cholesterol ester storage disease, LCAT deficiency)

Medications (anti-epileptics, HAART medications)

Reye's syndrome (mainly children)

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#### **Table 1-1. Rarer causes of hepatic steatosis**

These should be considered before diagnosing NAFLD (Masuoka and Chalasani, 2013). Key: HAART, highly active anti-retroviral drugs; HELLP syndrome, haemolysis elevated liver enzyme low platelets syndrome; HCV, hepatitis C virus; LCAT, lecithin cholesterol acyltransferase.

#### **1.1.2 Diagnosis and staging of NAFLD**

The identification of NAFLD remains a clinical challenge, as the majority of patients are asymptomatic, have non-specific signs until end-stage disease and often have unremarkable liver function tests (LFTs). This is particularly evident in primary care, in which there are no

validated tools for identifying those patients at risk of NASH and the advanced stages of fibrosis. Subsequently, the majority of referrals with suspected NAFLD from primary care to specialist liver units are based on incidental findings of abnormal LFTs and/or fatty liver on ultrasound (USS) (Dowman et al., 2011a). The sensitivity of abnormal liver enzymes (alanine aminotransferase (ALT) aspartate aminotransferase (AST), gamma glutamyltransferase (GGT)) is poor in detecting NAFLD, as a reported 55-79% of patients with NAFLD have levels within the normal reference ranges (Browning et al., 2004; Ratzliff et al., 2010a). Furthermore, the entire histological spectrum of NAFLD, including cirrhosis, can be observed in patients with normal liver enzymes (Adams et al., 2005c; Fracanzani et al., 2008). Subsequently, experts have questioned whether the normal reference ranges of serum aminotransferases need to be revised to increase the accuracy in NAFLD (Prati et al., 2002; Prati et al., 2005). Until new diagnostic tools are validated, a high degree of clinical awareness of the risk factors for NAFLD is required to determine who requires further investigation and follow-up.

#### *1.1.2.1 Clinical risk factors*

The most well established risk factors for NAFLD are insulin resistance (IR), obesity (especially central adiposity), type 2 diabetes, dyslipidaemia and arterial hypertension (Marchesini et al., 2001). Each of these abnormalities carries a risk of CVD, and collectively they are often categorised as the metabolic syndrome. In 2002, the third report of the National Cholesterol Education Program Expert Panel (NCEP)/ Adult Treatment Panel III (ATP III) provided a working definition of the metabolic syndrome, based on the combination of

central obesity, hypertension, hyperlipidaemia and hyperglycaemia (Table 1-2). Over 80% of patients with the metabolic syndrome have NAFLD, of which a quarter are found to have features of NASH on liver biopsy (Marceau et al., 1999). A Japanese prospective study (>4000 participants) highlighted that men and women with metabolic syndrome at baseline had an adjusted odds ratio of 4.0 and 11.2, respectively, for developing NAFLD over a 14 month period (Hamaguchi et al., 2005). Due to the strength of the association, NAFLD is widely recognised as the hepatic manifestation of the metabolic syndrome (Marchesini et al., 2001). Conversely, NAFLD and in particular NASH also have been shown to increase the risk of developing the metabolic syndrome and especially type 2 diabetes (Vanni et al., 2010).

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**Metabolic syndrome defined by 3 or more of the following:**

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Waist circumference >102 cm in men and >88 cm in women

Triglyceride concentration >1.7 mmol/L or on drug therapy for hypertriglyceridaemia

HDL cholesterol <1.03 mmol/L in men and <1.29 in women or on drug therapy for low HDL

Systolic BP  $\geq$ 130 mmHg or diastolic BP  $\geq$ 85 mmHg or on anti-hypertensive therapy

Fasting plasma glucose  $\geq$ 6.1 mmol/L or on anti-hyperglycaemic therapy

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**Table 1-2. ATP III definition of the metabolic syndrome (NCEP, 2002).**

Defined by NCEP/ATP III in 2002. Patients require  $\geq$ 3 components to be diagnosed with the metabolic syndrome. Key: BP, blood pressure; HDL, high density lipoprotein.

Type 2 diabetes is not only a risk factor for NAFLD, but increases the risk (2-4 fold) of progression to cirrhosis and HCC (Wong et al., 2010b; Wang et al., 2013b). Similarly, obesity has been shown to be an independent risk factor for carcinogenesis in NAFLD (El-Serag et al.,



2004; Kowdley and Caldwell, 2006; Wong et al., 2010b). Obesity studies imply that the higher the body mass index (BMI) the greater the prevalence of NAFLD (~90% in patients undergoing bariatric surgery) and severity of steatohepatitis (Marceau et al., 1999; De Ridder et al., 2007; Gholam et al., 2007). This correlation was also found in a large Italian general population study, using USS to define NAFLD (Bellentani et al., 2004). In particular, adipose distributed in the visceral/abdominal region appears to convey the greatest risk in NAFLD studies, as it has been shown to strongly correlate with the severity of hepatic steatosis (NB. measured on USS), irrespective of whether the individual was lean or obese (Eguchi et al., 2006). Following on from this, a small proof-of-concept study highlighted, with the use of parallel magnetic resonance imaging (MRI) and liver histology, that visceral adipose volume was an independent predictor of advanced NASH (Odds Ratio, OR 2.1) and fibrosis (OR 2.9), even after adjustment for IR and hepatic steatosis (van der Poorten et al., 2008). Other endocrine conditions have recently emerged as potential risk factors for NAFLD. These include polycystic ovarian syndrome, hypothyroidism, obstructive sleep apnoea, hypogonadism, and hypopituitarism (Vuppalanchi and Chalasani, 2009; Hazlehurst and Tomlinson, 2013).

In addition to metabolic risk factors, older age has been associated with higher prevalence of NAFLD, severe fibrosis and associated complications, including HCC (Adams et al., 2005b; Ascha et al., 2010). However, whether this is due to duration of the disease or independent features of ageing remains to be seen. Family members of patients with NAFLD have also at increased risk, independent of both age and obesity (Schwimmer et al., 2009).

### *1.1.2.2 Radiological diagnosis of NAFLD*

USS, computer tomography (CT) and MRI can be used to identify moderate to severe hepatic steatosis (Saadeh et al., 2002). USS is an accepted diagnostic tool for NAFL in primary/secondary care, as it is non-invasive, inexpensive and has no radiation exposure. In non-obese populations, the sensitivity and specificity are 60-90% and >90%, respectively (Masuoka and Chalasani, 2013). However, its sensitivity is limited in morbidly obese individuals and when the liver fat content is less than 33% (Saadeh et al., 2002). Furthermore, unlike CT and MRI it is operator-dependent with both inter- and intra-user variability. Non-contrast CT can be used to directly compare the attenuation of the liver to the spleen, in order to determine the presence of hepatic steatosis and to a certain degree estimate severity (sensitivity >80%) (Oliva et al., 2006; McKimmie et al., 2008). In the last 12 months, the Controlled Attenuation Parameter (CAP) scan has been developed by Echosens® (Paris, France) to quantify liver fat by the patient's bedside. This new modality, which is a modification of the more widely known Fibroscan® machine (i.e. measures liver stiffness), has shown early promise, but further validation is required (Myers et al., 2012; Sasso et al., 2012).

The current non-invasive gold standard, however, is proton magnetic resonance spectroscopy (H-MRS), which has repeatedly been shown to produce accurate quantitative measures of hepatic steatosis (sensitivity 88%, specificity 93%) (McPherson et al., 2009; Bohte et al., 2011). Due to the current cost of H-MRS and its limited availability, its uses at present are largely restricted to clinical research. To overcome this in part, Kotronen et al

devised two non-invasive scoring systems using a cohort of 470 subjects who had all undergone MRS (Kotronen et al., 2009). Both scores (detecting liver fat and % liver fat) incorporated the presence of metabolic syndrome, type 2 diabetes, fasting insulin, ALT and AST, with 86% sensitivity and 71% specificity (Kotronen et al., 2009). Similarly, the DIONYSOS study group developed the simpler fatty liver index, but this was developed using USS, which has a lower sensitivity than H-MRS (Bedogni et al., 2006).

Importantly, none of the above modalities can reliably identify or monitor the progression of NASH and/or the severity of fibrosis. Furthermore, there is the risk of false reassurance with negative imaging in patients with cirrhosis, as hepatic fat content loss can occur during disease progression to advanced fibrosis (Adams et al., 2005c).

#### *1.1.2.3 Liver biopsy*

USS-guided liver biopsy remains the reference method for the diagnosis of NAFLD, and more importantly for assessment of disease severity. Liver biopsy is the only validated tool for assessing the degree of necroinflammation, hepatocyte ballooning and for definitive staging of fibrosis (Chalasani et al., 2012). Therefore, at present it remains a necessity in selective clinical practice and therapeutic trials (Chapter 4; figure 4.2) for distinguishing simple hepatic steatosis ( $\pm$ mild inflammation) from active NASH (Sanyal et al., 2011). In clinical practice liver biopsy is reserved for patients deemed to be at risk of NASH and advanced fibrosis, and in whom there are competing aetiologies for hepatic steatosis (Table 1-1) and chronic liver disease (Chalasani et al., 2012).

In 1999, Brunt and colleagues designed the first histopathological classification for NASH, in order to assess the disease activity (i.e. grade) and the amount/pattern of fibrosis (i.e. stage) (Brunt et al., 1999). Six years later, Kleiner and colleagues from the NASH clinical research network in the United States (US) used this template to design the NAFLD Activity Score (NAS) (Kleiner et al., 2005). NAS is an unweighted sum (total of 8) of steatosis (0-3), inflammation (0-3), and hepatocytes ballooning (0-2); with the higher score representing greatest disease activity (*detailed in* Chapter 4; Table 4.1). In the same consortium they staged fibrosis using 4 categories, ranging from no fibrosis (F0) through to bridging fibrosis and cirrhosis, which are termed F3 and F4, respectively (Kleiner et al., 2005). Many authors collectively refer to the latter stages (F3-F4) as advanced fibrosis. The sole purpose of the NAS was to define and quantify disease activity in therapeutic clinical trials of NASH. However, It is important to recognise that there is no reliable NAS cut-off that can be used to definitively diagnose NASH (Brunt et al., 2011). Therefore, experts believe that an accurate diagnosis of definite NASH (or *borderline* NASH) should be based on pattern recognition rather than composite scoring systems. The different terminologies are summarised in (Table 1-3).

NASH as a single disease entity incurs a greater risk of developing end-stage liver disease and liver-related mortality than those with simple hepatic steatosis ( $\pm$  mild inflammation) (Matteoni et al., 1999; Ekstedt et al., 2006; Söderberg et al., 2010). In contrast, the predictive value of NAS remains unknown. Subsequently, an expert trials consortium in 2011 recommended that the reversal of NASH (with no worsening fibrosis) in therapeutic trials of

short-duration (<2 years), is a better surrogate of reduced liver-related mortality than changes in NAS alone (Sanyal et al., 2011).

<b>Term</b>	<b>Steatosis*</b>	<b>Hepatocyte ballooning</b>	<b>Lobular inflammation</b>	<b>Pattern/zonality of liver lesions</b>
<b>NAFL</b>	Macrovesicular	Absent	Present or absent	Any pattern
<b>NASH</b>				
<b>Borderline (type 1)</b>	Macrovesicular	Absent	Present or absent	Acinar or pan-acinar zone 1
<b>Borderline (type 3)</b>	Macrovesicular	Absent	Present	Acinar zone 3
<b>Definite</b>	Macrovesicular	Present** (mild-severe)	Present	Predominant centrilobular, acinar zone 3

**Table 1-3. Histological classification of NAFL and NASH.**

\*Hepatic steatosis is defined as >5% macrovesicular steatosis on light microscopy.

\*\*‘Classical’ hepatocyte ballooning (pale hepatocytes with apoptotic bodies) can be confirmed with ubiquitin immunohistochemistry. Key: NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis. Table is an adaption of (Sanyal et al., 2011).

Although liver biopsy remains strongly recommended in the majority of clinical trials of NASH, it is not without its limitations. Most notably, its invasive and thus carries a risk of morbidity (i.e. haemorrhage) and very rarely mortality (<1/10,000) (Bravo et al., 2001). Therefore, there is an understandable reluctance of patients to undergo repeated procedures for disease assessment. Furthermore, it is subject to sampling error (i.e. 1/50,000<sup>th</sup> proportion of the liver is sampled) (Ratziu et al., 2005) and inter-/intra-pathologist variability (Brunt et al., 2011; Sanyal et al., 2011). The latter, of which was exemplified in the PIVENS trial of pioglitazone and vitamin E (Sanyal et al., 2010). These can be minimised to a certain extent by biopsy technique (needle size, orientation) and the use of at least two independent histopathologists (Sanyal et al., 2011).

#### *1.1.2.4 Non-invasive tools for identification of NASH and fibrosis*

In light of the limitations of liver biopsy, there has been an intense interest in non-invasive markers for a) the diagnosis of NASH, b) staging fibrosis and c) assessing the efficacy of treatments of NASH (Rockey and Bissell, 2006). These range from simple non-invasive scoring systems (e.g. NAFLD Fibrosis Score [NFS]), complex serum biomarkers (e.g. Enhanced Liver Fibrosis [ELF] test), through to novel imaging tools (e.g. transient elastography); all of which have been extensively reviewed in the literature (Guha et al., 2006; Rockey and Bissell, 2006; Dowman et al., 2011b; Musso et al., 2011). The non-invasive markers that have been studied and validated in large cohorts of NAFLD (vs. biopsy) are summarised in (Table 1-4).

##### **1.1.2.4.1 SIMPLE NON-INVASIVE SCORING SYSTEMS**

Several noninvasive scoring systems have been developed to distinguish between patients with and without advanced NAFLD fibrosis. These include the NFS (Angulo et al., 2007), BARD (Harrison et al., 2008), Fib-4 (Vallet-Pichard et al., 2007), AST/platelet ratio index (APRI) (Wai et al., 2003) and more simply the AST/ALT ratio (Williams and Hoofnagle, 1988). With the exception of the NFS and BARD scores, most were originally designed to identify advanced fibrosis in viral hepatitis or non-specified liver disease. However, all have been externally validated in secondary care populations of NAFLD (McPherson et al., 2010; Musso et al., 2011).

	Description	Diagnosis	Key References
<b>Simple Scoring systems</b>			
<b>NAFLD Fibrosis Score (NFS)</b>	Age, BMI, AST/ALT, albumin, platelet count, Type 2 diabetes/IFG	Advanced fibrosis	(Angulo et al., 2007) (McPherson et al., 2010)
<b>BARD</b>	BMI, AST/ALT, Type 2 diabetes	Advanced fibrosis	(Harrison et al., 2008) (McPherson et al., 2010)
<b>Fib-4</b>	Age, AST, ALT, platelet count	Advanced fibrosis	(Vallet-Pichard et al., 2007) (McPherson et al., 2010)
<b>AST/ALT ratio</b>	AST, ALT	Advanced fibrosis	(Williams and Hoofnagle, 1988) (McPherson et al., 2010)
<b>AST/platelet ratio index (APRI)</b>	AST, platelet count	Advanced fibrosis	(Wai et al., 2003) (McPherson et al., 2010)
<b>Fibrometer</b>	Age, weight, AST, ALT, platelet count, glucose, ferritin	Advanced fibrosis	(Calès et al., 2009)
<b>Complex Serum biomarkers</b>			
<b>CK-18 fragments</b>	Serum marker of hepatocyte apoptosis (M30 fragment)	Steatohepatitis (diagnostic cut-off not established)	(Feldstein et al., 2009b)
<b>NASH test</b>	AST, total cholesterol, triglycerides, fasting glucose, weight, and height (+Fibrotest)	Steatohepatitis	(Poynard et al., 2006) (Poynard et al., 2012)
<b>Enhanced Liver Fibrosis (ELF)</b>	Serum levels of three matrix turnover proteins: hyaluronic acid, TIMP-1, P3NP	Fibrosis stage (no/mild; moderate; severe/cirrhosis)	(Rosenberg et al., 2004) (Guha et al., 2008)
<b>Fibrotest</b>	Age, gender, GGT, apolipoprotein A1, haptoglobin, $\alpha$ -2 macroglobulin.	Advanced fibrosis (correlates with stage)	(Ratzu et al., 2006)
<b>Hepascore</b>	Age, gender, bilirubin, GGT, hyaluronic acid, $\alpha$ -2 macroglobulin.	Advanced fibrosis (correlates with stage)	(Adams et al., 2005a) (Adams et al., 2011)
<b>Imaging tools</b>			
<b>Transient Elastography (Fibroscan®)</b>	USS-based measurement of liver stiffness (via mechanical vibration waves)	Advanced fibrosis (cut-offs not determined for F0-F4 staging). NB. Not available in US.	(Wong et al., 2010a)
<b>Acoustic Radiation Force Impulse (ARFI)</b>	USS-based measurement of liver stiffness by the bed-side (via radiation forces)	Advanced fibrosis (limited availability at present)	(Yoneda et al., 2010)
<b>MR Elastography</b>	MR imaging (via mechanical vibration)	Advanced fibrosis (limited availability at present)	(Kim et al., 2013b)

**Table 1-4. Summary of the non-invasive markers of NASH and advanced fibrosis.**

The non-invasive markers include simple scoring systems (i.e. routine laboratory tests), complex serum biomarkers and imaging modalities. Key (*infrequent abbreviations only*): P3NP, type III procollagen peptide; TIMP-1, tissue inhibitor of metalloproteinase 1.

In 2010, McPherson and colleagues compared the diagnostic performance of the five non-invasive scoring systems using a well-characterised secondary care cohort of 145 obese patients with biopsy-proven NAFLD (McPherson et al., 2010). In doing so they identified that all five scores (including AST/ALT ratio) can reliably exclude advanced fibrosis, with negative predictive values (NPV) above 90% throughout. The positive predictive values (PPV), using the highest cut-off per test, were modest in all cases (PPV 27-79%), with Fib-4 (cut-off  $>+3.25$  = PPV 75%) and NFS (cut-off  $>+0.676$  = 79%) performing the best (McPherson et al., 2010). However, as the PPV were sub-optimal, a liver biopsy is still recommended to confirm a high score in any case. The same authors have recently validated the NFS in patients with NAFLD and normal liver enzymes, and highlighted similar levels of accuracy (McPherson et al., 2013). NFS is based on age, BMI, presence of type diabetes (or impaired fasting glucose (IFG)), platelet count, albumin, and the AST/ALT ratio. On its own the formula is complex, yet easy to use calculators exist online for clinical application (<http://naflscore.com>). The NFS (and Fib-4) may be of clinical use in the primary care setting, as they are simple to use, cheap and incorporate routine clinical and laboratory variables. However, no UK studies prior to my thesis had applied them in this setting.

#### 1.1.2.4.2 COMPLEX SERUM BIOMARKERS

The ELF panel and the Fibrotest are the most widely researched serum biomarkers for fibrosis in the field of NAFLD. Despite being commercially available in the UK, they have not been fully adopted by the National Health Service (NHS) and are largely used for research purposes at present. Rosenberg and colleagues developed the serum ELF test in 2004 using a



panel of three serum matrix turnover proteins (Rosenberg et al., 2004) (Table 1-4). It has since been validated in an independent cohort of 192 patients with biopsy-proven NAFLD and found to have a sensitivity of 80% and specificity of 90% for identifying advanced fibrosis (cut-off  $>+0.358$ ) (Guha et al., 2008). Unlike most serum biomarkers, the ELF has commercially available cut-offs for no/mild, moderate and severe fibrosis. The Fibrotest, which originated from a French research group in 2006, incorporates routine clinical variables (gender, age, GGT) and complex biomarkers of fibrosis, namely apolipoprotein A1, haptoglobin, and  $\alpha$ -2 macroglobulin (Ratzu et al., 2006). In keeping with the ELF panel, it has been validated in a large cohort ( $>400$  patients) with similar diagnostic accuracy (Poynard et al., 2012). It is noteworthy, however, that these tests have mainly been validated in morbidly obese cohorts and therefore their accuracy may not translate to the general population or primary care.

To date, fewer serum biomarkers have been validated for the identification of active NASH (Poynard et al., 2006; Feldstein et al., 2009b; Joka et al., 2012; Poynard et al., 2012) (Table 1-4). Cytokeratin-18 (CK-18) fragment (M30, and more recently M65 (Joka et al., 2012)) has emerged as a promising serum biomarker for active steatohepatitis over the last 5 years. In particular, CK-18 M30 fragment, which is released into the circulation with hepatocyte apoptosis (i.e. after caspase 3 cleavage), have been validated in multi-centre studies (Feldstein et al., 2009b; Shen et al., 2012). In a study of 139 patients with histological NAFLD, Feldstein et al found that CK-18 M30 was an independent predictor of NASH, after adjustment for confounders such as liver enzymes, age and fibrosis (Feldstein et al., 2009b). This has been externally validated by a recent meta-analysis, highlighting a sensitivity and

specificity of 78% and 87%, respectively (Musso et al., 2011). At present, however, CK-18 remains a research tool in many countries and there are no established reference ranges for clinical use.

#### 1.1.2.4.3 IMAGING TOOLS FOR ADVANCED FIBROSIS

MR elastography (Kim et al., 2013b) and Acoustic Radiation Force Impulse (ARFI) (Yoneda et al., 2010) are both promising non-invasive imaging tools for the assessment of liver fibrosis, but their cost and required expertise has currently restricted their use to a few specialist research units. In contrast, several large non-UK meta-analyses (>1000s patients) have confirmed the diagnostic accuracy of transient elastography (commercial name Fibroscan®, Echosens, France) in NAFLD and a variety of other chronic liver diseases (Talwalkar et al., 2007; Friedrich-Rust et al., 2008; Yoneda et al., 2010; Tsochatzis et al., 2011). Transient elastography uses a modified USS probe to measure the velocity of an elastic shear wave (*created by a vibration source in the probe*), which in turn provides a painless, quick (<10 mins) evaluation of liver stiffness (in kPa) at the patient's bedside. As of May 2013, there were over 130 Fibroscan machines in use in the UK and Ireland (Armstrong et al., 2013b). In one of the largest studies to date in NAFLD (n=246), Wong et al reported that transient elastography (cut-off of 7.9 kPa) has a PPV of 52% and a NPV 97% for advanced fibrosis (Wong et al., 2010a). The latter value highlights that it is an excellent exclusion tool for advanced fibrosis in NAFLD, but the moderately low PPV was attributed to the use of the older medium-sized 'M'-probe in obese individuals. This was partly rectified with the

introduction of larger 'XL'-probe in 2010, but patients with a BMI >35 kg/m<sup>2</sup> were still 9 times more likely to have an inaccurate fibrosis reading (Wong et al., 2012b).

Collectively the data highlights that non-invasive markers of fibrosis are promising, but at present are not robust enough to replace liver biopsy as the primary outcome measure in trials or for diagnostic confirmation in clinical practice (Sanyal et al., 2011). This is largely due to the fact that the majority of non-invasive markers have been investigated in cross-sectional studies in NAFLD, thereby limiting our knowledge with regards to their predictive accuracy for natural disease progression and for resolution after therapeutic intervention.

### **1.1.3 Epidemiology of NAFLD**

#### *1.1.3.1 Incidence of NAFLD*

Very few studies have estimated the incidence of NAFLD, with estimates ranging from 18.5 to 85 per 1000 person years (Hamaguchi et al., 2005; Suzuki et al., 2005; Bedogni et al., 2007; Whalley et al., 2007). These estimates are far from conclusive, however, as they are from selected populations (i.e. secondary care referrals in UK study (Whalley et al., 2007)), rely on surrogate markers on NAFLD (i.e. abnormal ALT (Suzuki et al., 2005)) and as was the case of sub-group analysis from the Italian DIONYSOS study, failed to exclude excess alcohol consumption (Bedogni et al., 2007). Furthermore, due the fact these cohorts pre-date back to 2002-2003 and the incidence of risk factors for NAFLD (type 2 diabetes, obesity) has risen exponential since this time period, they likely underestimate the current incidence.

#### *1.1.3.2 Prevalence of NAFLD*

The reported prevalence of NAFLD ranges from 14 to 31% in general population studies from Europe (Bedogni et al., 2005; Caballería et al., 2007), Asia (Hamaguchi et al., 2005) and the US (Browning et al., 2004). The marked variation between studies likely reflects ethnic diversity (Browning et al., 2004; Petersen et al., 2006), the underlying risk profile of the populations (i.e. rates of obesity) and most notably, differences in NAFLD classification. Two of the largest (n>2000) and likely most representative studies originate from the US (Dallas Heart Study) and Italy (DIONYSOS study) (Browning et al., 2004; Bedogni et al., 2005). The DIONYSOS study was a large, prospective study designed to investigate the prevalence of chronic liver disease in two northern Italian towns (Bellentani et al., 1994). After exclusion of viral hepatitis and alcohol excess (>20g/day both sexes), the prevalence of NAFLD on USS was 25% and 20% in patients with and without elevated liver enzymes, respectively (Bedogni et al., 2005). Using the more sensitive technique of H-MRS (NAFLD >5.6% liver fat), the prevalence was as high as 31% in the ethnic diverse population (n=2287) of the US Dallas Heart Study (Browning et al., 2004). Both population studies also highlighted that the prevalence can be as high as 17% in lean individuals (Bellentani et al., 2000; Browning et al., 2004). Prevalence rates rise significantly in selected high-risk populations. Studies highlight that as many as 70% of patients with type 2 diabetes and 90% in those undergoing bariatric surgery have USS and/or histological defined NAFLD (Marceau et al., 1999; De Ridder et al., 2007; Gholam et al., 2007; Targher et al., 2007; Williams et al., 2011).

As patients with NASH are considered to carry the highest risk of disease progression, understanding the true clinical burden of NAFLD in population studies remains a challenge. At present, there is a paucity of data on the prevalence of NASH and advanced fibrosis in primary care and the general population. The majority of our estimates in this setting have to be taken from living-related liver donor datasets, in which the reported prevalence of biopsy-defined NASH range from 1.1% in Japan (Yamamoto et al., 2007) through to 5-16% in the UK and US (Ryan et al., 2002; Nadalin et al., 2005; Minervini et al., 2009). However, as alcohol consumption was not reported in these studies, world-wide estimates are thought to be closer to 5% (Argo and Caldwell, 2009). Reported estimates, however, rise up to 22-33% in patients with type 2 diabetes and/or severe obesity (BMI >35 kg/m<sup>2</sup>) (Beymer et al., 2003; Boza et al., 2005; Ong et al., 2005; Williams et al., 2011). In the latter, the rate of incidental advanced fibrosis/cirrhosis is 1.6%. With the exception of selected NAFLD patients attending specialist liver units (Hultcrantz et al., 1986; Daniel et al., 1999; Skelly et al., 2001; Ekstedt et al., 2006; Angulo et al., 2007; Neuschwander-Tetri et al., 2010; Söderberg et al., 2010), data on the prevalence of advanced fibrosis is lacking. Future studies should look to utilise non-invasive markers (*as discussed above*) to broaden our knowledge with regards to the prevalence of advanced fibrosis in unbiased populations, in which liver biopsy is not deemed appropriate (i.e. primary care).

#### **1.1.4 Pathogenesis of NAFLD**

Over the last two decades, several pathophysiological mechanisms have been proposed for the development of hepatic steatosis and subsequent progressive liver injury and fibrosis in

NAFLD (Day and James, 1998; Day, 2002; Dowman et al., 2010; Cohen et al., 2011; Cusi, 2012). Despite several advances in the pathogenesis of hepatic steatosis, it still remains unclear why some individuals (*minority*) develop steatohepatitis, cirrhosis and liver failure, whilst others with similar metabolic risk profiles do not progress beyond simple hepatic steatosis. Experts believe that a complex interplay between genetic susceptibility and multiple environmental factors contribute to progressive disease (Day, 2002), but identification of a key causative factor amongst the mist of overlapping metabolic diseases remains a challenge. However, in light of the robust epidemiological, therapeutic, and recent euglycaemic clamp evidence, there is uniform agreement amongst the field that IR plays a critical role in the pathogenesis in NAFLD.

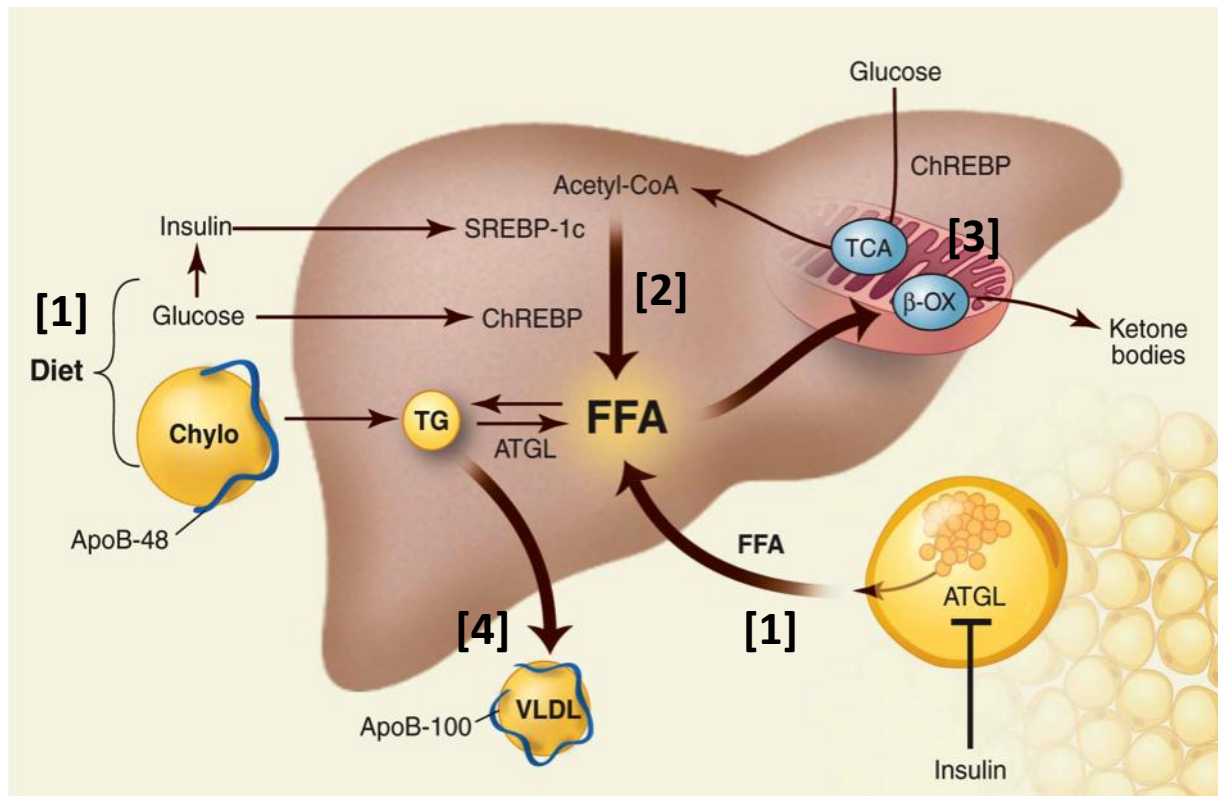
#### *1.1.4.1 Pathogenesis of hepatic steatosis*

Hepatic steatosis (or NAFL) is defined by excess triglyceride accumulation in hepatocytes, which arises from an imbalance between triglyceride synthesis (+uptake) and utilisation (+export). Triglycerides are formed from the esterification of three non-esterified fatty acids (NEFA) to a glycerol backbone. The liver has three sources of NEFA (Cohen et al., 2011), which include:

- ❖ Diet: Dietary fats are taken up by the intestine and packaged into chylomicrons for delivery to the systemic circulation. The majority of the triglyceride in the chylomicrons is hydrolysed to release NEFA for peripheral uptake. However, approximately 20% is delivered directly to the liver (Cohen et al., 2011).

- ❖ De novo lipogenesis (DNL): The intake of carbohydrate (glucose) increases the availability of insulin and acetyl-coenzyme A (CoA), which is the main substrate for *de novo* synthesis of NEFA. Insulin stimulates sterol regulatory element binding protein-1c (SREBP-1c), whereas glucose stimulates carbohydrate responsive element-binding protein (ChREBP). Both of these transcription factors promote DNL, via activation of key rate-limiting enzymes, namely acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (Postic and Girard, 2008; Cohen et al., 2011).
  
- ❖ Adipose tissue: NEFA are released from adipose tissue in the fasting and IR states via lipolysis. Lipolysis is the hydrolysis of NEFA and glycerol from triglyceride.

In 2005, Donnelly et al shed major light on the relative contribution of each of these sources on hepatocyte triglyceride accumulation in NAFLD (Donnelly et al., 2005). Using multiple stable isotope tracers in parallel to liver biopsies, they demonstrated that 59% of hepatocyte triglycerides (in the disease state) were derived from the adipose tissue, 26% from hepatocyte DNL and the remainder from the diet (Donnelly et al., 2005). In addition to excess NEFA delivery and hepatic DNL, hepatic steatosis can occur as a result of decreased NEFA metabolism ( $\beta$ -oxidation) and/or decreased NEFA export from hepatocytes (Postic and Girard, 2008) (Figure 1-1).



**Figure 1-1. Mechanisms of excess triglyceride accumulation in NAFLD.**

Excess triglyceride accumulation in the liver ('hepatic steatosis') can occur as a result of four abnormalities: [1] Excess delivery of FFA via adipose lipolysis (with adipose insulin resistance) and/or dietary excess; [2] Excess endogenous synthesis via DNL; [3] decreased FFA breakdown ( $\beta$ -oxidation in the mitochondria); and/or [4] decreased export via packaging with apolipoprotein B (Apo-B) into VLDL. Insulin inhibits lipolysis in adipose tissue by suppressing adipose triglyceride lipase (ATGL)/hormone sensitive lipase. Other key abbreviations: Chylo, chylomicron; ChREBP, Carbohydrate-responsive element-binding protein;  $\beta$ -OX,  $\beta$ -oxidation; SREBP-1c, sterol regulatory element-binding protein; TCA, tricarboxylic acid cycle (Krebs cycle). NB. FFA, free fatty acids = NEFA for purposes of the figure. Figure has been adapted from the original, which is from (Cohen et al., 2011).

#### 1.1.4.2 Two-hit hypothesis

In 1998, Day and James proposed the 'two-hit hypothesis' for the pathogenesis of NAFLD, in which the primary insult is hepatic steatosis ('first hit') (Day and James, 1998). This in turn, makes the liver more susceptible to injury mediated by 'second hits,' such as inflammatory



cytokines/adipokines (e.g. tumour necrosis factor-alpha [TNF- $\alpha$ ]), mitochondrial dysfunction (adenosine triphosphate [ATP] depletion) and oxidative stress (lipid peroxidation) (Day and James, 1998; Day, 2002). Together, cytokine-mediated injury and oxidative stress-induced lipid peroxidation act as key mediators of necroinflammation and fibrosis (via stellate activation) in NASH (Day, 2002). Leading on from this, others have proposed a 'third hit,' whereby impaired liver regeneration (e.g. impaired hepatocyte 'oval' progenitor cell proliferation) in response to hepatocyte apoptosis occurs (Jou et al., 2008; Dowman et al., 2010).

#### *1.1.4.3 Lipotoxicity*

The theory that hepatic steatosis is the primary insult ('first' hit) in NAFLD has since been questioned, as a result of a recent expansion of evidence surrounding the relationship ('cross-talk') between the liver and adipose tissue. Indeed, there is now strong evidence to indicate that circulating NEFA and their metabolic by-products (diacylglycerol/triacylglycerol) induce direct lipotoxic injury to key metabolic organs, including the pancreas, skeletal muscle and most notably, the liver (Belfort et al., 2005; Cusi et al., 2007; Malhi and Gores, 2008; Kotronen et al., 2010). Based on the fact that the majority of NEFA originates from the adipose in NAFLD, it is now the current belief that the initial insult occurs in the adipose tissue.

The initial process in NAFLD development is believed to be the result of the expansion of adipose tissue (hypertrophy), secondary to putative environmental/dietary factors (i.e.

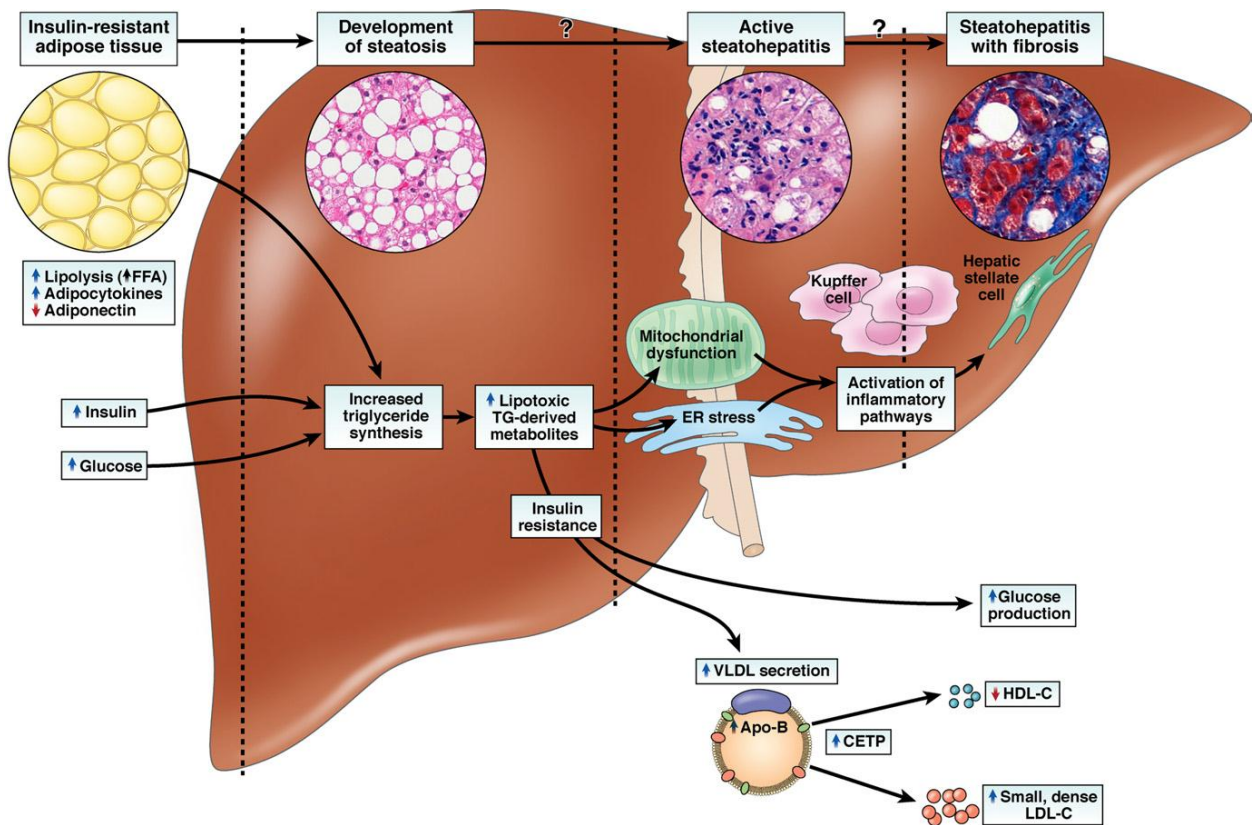
overfeeding) and underlying genetic susceptibility (Cusi, 2012). Hypertrophic adipose tissue fails to then cope with the chronic energy supply and several localised inflammatory pathways are activated within the tissue (e.g. inhibitor  $\kappa$ B kinase/nuclear factor  $\kappa$ B and c-Jun N-terminal kinase/activator protein 1 pathways (Yuan et al., 2001; Zhang et al., 2011). In addition, the dysfunctional adipose tissue releases key mediators into the circulation (i.e. monocyte chemoattractant protein 1 [MCP-1]), which are known to increase macrophage recruitment to the adipose tissue. Collectively, these result in adipose inflammation and subsequent localised IR (Cusi, 2012). Of note, in an adipose insulin-sensitive state, insulin stimulates triglyceride production (from NEFA + glycerol) via inactivation of hormone sensitive lipase and therefore switches off lipolysis. In contrast, adipose IR results in uncontrolled lipolysis, with subsequent flux of excess NEFA into the circulation and the liver.

Accompanying NEFA into the systemic system is a plethora of pro-inflammatory cytokines (known as adipocytokines) from the adipose tissue (Cusi, 2012), which include TNF $\alpha$  and interleukin-6 (IL-6). Both of these cytokines are increased in patients with obesity, IR (Kern et al., 2001) and NASH (Grigorescu et al., 2012). In contrast, adiponectin, which is produced by mature adipocytes and has both insulin sensitising and anti-inflammatory properties, is reduced in patients with NAFLD (Polyzos et al., 2010). These cytokine profiles provide further support that adipose metabolic dysfunction is an integral part of NASH.

The lipotoxic injury that occurs as a result of NEFA flux to the liver and other metabolically active organs is termed 'lipotoxicity' (Figure 1-2). Lipotoxicity leads to increased hepatic glucose production (i.e. hepatic IR), decreased muscle glucose uptake (muscle IR), and

impaired insulin clearance (Sanyal et al., 2001; Bugianesi et al., 2005a; Gastaldelli et al., 2009; Lomonaco et al., 2011; Musso et al., 2012). The resultant effect is hyperglycaemia in the face of hyperinsulinaemia, which are hallmarks of systemic IR in NASH. The combination of hyperinsulinaemia and increased NEFA flux results in excess hepatocyte triglyceride accumulation (Donnelly et al., 2005). In addition to steatosis, several different mechanisms have been proposed for how lipotoxicity causes progressive hepatocyte injury in NASH. These include hepatocyte apoptosis ('lipoapoptosis'), mitochondrial dysfunction, defective macroautophagy (i.e. removal of cell debris), Kupffer cell and other immune cell activation, and perpetuating cytokine release (Malhi and Gores, 2008; Amir and Czaja, 2011; Grattagliano et al., 2012; Ono and Saibara, 2012). Collectively, these likely contribute to hepatic stellate cell activation and progressive fibrosis, but these mechanisms require more extensive research.

Despite these advances in IR and adipose tissue biology, only a handful of studies to date have assessed organ-specific IR in the context of NASH (Sanyal et al., 2001; Bugianesi et al., 2005a; Gastaldelli et al., 2009; Lomonaco et al., 2011; Musso et al., 2012). Furthermore, gaps in our knowledge exist with regards to what types of adipose depot (i.e. visceral, abdominal subcutaneous, peripheral) exhibit the dysfunctional properties described above and may in turn, act as future therapeutic targets for NASH.



**Figure 1-2. Proposed mechanism for the role adipose tissue dysfunction in NAFLD (Cusi, 2012).**

Overview of how adipose IR and lipolysis can trigger excess triglyceride accumulation in the liver and a cascade of hepatic dysfunction. The proposed effect by Cusi et al includes hepatic IR, oxidative stress, activation of numerous inflammatory pathways and fibrogenesis via activation of stellate cells. Proposed mechanisms for the progression of hepatic steatosis through to steatohepatitis and fibrosis still require further research. Key: Apo-B, apolipoprotein B; CETP, Cholesteryl ester transfer protein; ER, endoplasmic reticulum; FFA, free fatty acids; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglyceride; VLDL, very low density lipoprotein.

### 1.1.5 Natural history and complications of NAFLD

Patients with NAFLD (i.e. the whole spectrum) have a 70% higher risk of mortality than the general population of similar age and sex (Adams et al., 2005b; Ekstedt et al., 2006;

Söderberg et al., 2010). The vast majority of deaths are attributed to CVD, followed by malignancy and liver failure. The long-term prognosis for patients diagnosed with NAFLD varies markedly across the spectrum of the disease (Musso et al., 2011). Longitudinal follow-up studies, spanning 5 to 21 years duration, have emerged in recent years to expand our knowledge of the natural disease course of NAFLD and in particular NASH (Matteoni et al., 1999; Dam-Larsen et al., 2004; Adams et al., 2005b; Ekstedt et al., 2006; Rafiq et al., 2009; Söderberg et al., 2010). In general patients with NAFL (i.e. simple steatosis and negligible inflammation) are widely believed to have a slow, relatively benign disease, with less than 1% progressing to cirrhosis over an average of 15 years follow-up (Angulo, 2010). In contrast, approximately 4-14% of patients with NASH will progress to cirrhosis over the same time course (Angulo, 2010), with age and liver inflammation being the main risk factors for such (Argo et al., 2009). Consequently, patients with NASH have significantly higher risk of liver-related death than those with simple steatosis (odds ratio 5.7). This risk is even more pronounced for those with NASH and advanced fibrosis (odds ratio 10) (Musso et al., 2011). Indeed, those with advanced NASH fibrosis are at increased risk of HCC, with a yearly cumulative incidence of 2.6% (vs. 4.6% in HCV) (Sanyal et al., 2006). The fact that approximately 3-5% of the population are estimated to have NASH, it is not surprising that it is expected to become the commonest indication for liver transplantation in the next few years (Charlton et al., 2011).

Recent evidence also highlights that the clinical burden of NASH is not restricted to liver morbidity, as it incurs an increased risk of incident CVD, type 2 diabetes (adjusted 2-5 fold risk), chronic kidney disease (adjusted 1.5-2 risk) and malignancy (i.e. colorectal cancer).

Furthermore, there is mounting evidence from prospective studies to imply that the risk of such is independent of associated metabolic factors (i.e. smoking, age, obesity). Further detail of these extra-hepatic risks of NASH is summarised in our review in the Appendix (Armstrong et al., 2013a).

### **1.1.6 Management of NAFLD**

The management of patients with NAFLD requires a multi-disciplinary approach to enable treatment of the underlying liver disease as well as the associated metabolic co-morbidities (Armstrong et al., 2013c; Cobbold et al., 2013). It is currently recommended that treatment options for NAFLD should be reserved for patients with NASH, who are at higher risk of disease progression (Chalasani et al., 2012). In particular, the focus should be on patients who have been unable to achieve or maintain weight loss with lifestyle intervention.

#### *1.1.6.1 Lifestyle interventions*

Lifestyle modification remains at the cornerstone of treatment for NAFLD and its associated CVD risk factors. Several studies indicate that diet alone or in combination with exercise can improve IR, liver enzymes and hepatic steatosis (average 40% reduction) on H-MRS (Chalasani et al., 2012; Cusi, 2012). However, large long-term randomised-controlled trials (RCT) investigating the histological effect of lifestyle intervention programmes are currently lacking.

The most robust RCT to date was performed by Promrat and colleagues in 31 overweight or obese patients with biopsy-proven NASH (Promrat et al., 2010). Patients were either randomised to 48 weeks of structured education (control) or intensive lifestyle intervention, consisting of diet, behavioural advice and moderate physical activity (200 mins/week). The intervention arm achieved 9.3% weight loss compared to 0.2% in the controls. Most notable, was the fact that patients with  $\geq 7\%$  weight loss had significant improvements (vs.  $< 7\%$  weight loss) in hepatic steatosis, necroinflammation and hepatocyte ballooning (Promrat et al., 2010). Albeit via a different strategy, Harrison et al found that  $\geq 5\%$  weight loss over 36 weeks resulted in reduced IR and hepatic steatosis, and only those that lost  $\geq 9\%$  of their body weight achieved histological improvements consistent with the Promrat study (Harrison et al., 2009). Neither study reported improvements in fibrosis, which is likely attributed to the short-duration of follow-up. A recent prospective study with paired liver biopsies showed, however, that sustained weight loss over 3 years prevents progression of fibrosis in NASH (Wong et al., 2010b).

#### *1.1.6.2 Surgical options*

To date, two large meta-analyses have evaluated the influence of bariatric surgery on the histological features of NASH. The analysis by Mummadi et al (15 studies; 766 paired biopsies) found that over two-thirds of patients undergoing bariatric surgery had improvements in all the components of NASH (including fibrosis) by one year (Mummadi et al., 2008). In contrast, Chavez-Tapia et al in 2010 concluded that there was insufficient high quality evidence (i.e. no RCTs) to accurately weigh up the benefits in comparison to the risks

of bariatric surgery in patients with NASH. Indeed, out of the 21 studies they reviewed, four reported a deterioration in fibrosis (Chavez-Tapia et al., 2010). Overall, this culminated in the authors of the recent US guidelines (AGA/AASLD) concluding that is premature to consider bariatric surgery as a targeted treatment option for NASH at present (Chalasani et al., 2012).

#### *1.1.6.3 Pharmacological options for NASH*

In total, there are less than thirty well designed RCTs (i.e. powered, paired liver biopsies) in the literature that have investigated the histological efficacy of potential pharmacological therapies in patients with NASH. Due to the nature of the disease, the majority of these have focused on insulin sensitisers (e.g. metformin, thiazolidinediones [TZD]), anti-oxidants (e.g. vitamin E) and weight loss therapies (e.g. orlistat). Fewer RCTs have focused on lipid-lowering (e.g. statins), anti-inflammatory (e.g. anti-TNF $\alpha$  agents) and anti-fibrotic agents (e.g. angiotensin receptor blockers) (Table 1-5). An in depth discussion of these trials, undertaken by myself and others, is included in the Appendix (Dowman et al., 2011a).



Therapy	Proposed mechanism of action	Key references only
<b>Insulin sensitising agents</b>		
<b>Metformin</b>	Insulin sensitisation (hepatic > skeletal muscle) via AMPK activation. NB. Not definitive.	(Uygun et al., 2004) (Bugianesi et al., 2005b) (Idilman et al., 2008) (Haukeland et al., 2009) (Shields et al., 2009)
<b>Thiazolidinediones</b> - Pioglitazone - Rosiglitazone*	Insulin sensitisation via peroxisome proliferator-activated receptors gamma (PPAR $\gamma$ ) agonism (mainly in adipose tissue).	(Belfort et al., 2006) (Ratziu et al., 2008) (Aithal et al., 2008) (Ratziu et al., 2010b) (Sanyal et al., 2010) (Torres et al., 2011)
<b>Weight losing agents</b>		
<b>Orlistat</b>	Pancreatic/gastric lipase inhibitor, which inhibits the absorption of dietary triglycerides.	(Zelber-Sagi et al., 2006) (Harrison et al., 2009)
<b>Anti-oxidants</b>		
<b>Vitamins</b> - Vitamin E - Vitamin C - Betaine - L-carnithine	Reduces intracellular oxidative stress. Mechanisms remain unclear.	(Harrison et al., 2003) (Dufour et al., 2006) (Nobili et al., 2008) (Abdelmalek et al., 2009) (Sanyal et al., 2010) (Malaguarnera et al., 2010)
<b>Lipid lowering therapies</b>		
<b>Statins</b>	Increases HDL and lowers LDL via 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibition	(Nelson et al., 2009)
<b>Hepato-protective/anti-inflammatory agents</b>		
<b>Ursodeoxycholic acid</b>	Bile/Lipid metabolism TNF- $\alpha$ inhibition?	(Lindor et al., 2004) (Dufour et al., 2006) (Leuschner et al., 2010)
<b>Pentoxifylline</b>	Direct cytoprotective actions? TNF- $\alpha$ inhibition?	(Van Wagner et al., 2011) (Zein et al., 2011)
<b>Anti-fibrotic agents</b>		
<b>Angiotensin receptor blockade</b> - Telmisartan/Valsartan (NB no RCT in losartan)	Decrease activation of hepatic stellate cells. Insulin-sensitising effects. Mechanisms not fully explained.	(Georgescu et al., 2009)

**Table 1-5. Summary of pharmacological agents that have been trialed in NASH.**

Table only includes the main randomized-controlled trials and those that have histological end-points (up until 2012). \*Rosiglitazone withdrawn from Europe in 2010 due to safety concerns (cardiovascular side effects).

#### 1.1.6.3.1 WEIGHT LOSING AGENTS

Two RCTs have investigated the effect of orlistat, an oral pancreatic lipase inhibitor, in patients with biopsy-proven NASH. Although the earlier trial by Zelber-Sagi et al offered encouraging results with regards to reductions in ALT and hepatic steatosis on USS, it failed to comment on histology due to a high biopsy refusal rate at the end of treatment (Zelber-Sagi et al., 2006). In contrast, a more recent, adequately powered study (n=50) failed to show an additional histological benefit with 36 weeks therapy with orlistat compared to placebo (Harrison et al., 2009). Other weight loss agents, namely sibutramine and rimonabant, have been taken off the market as a result of cardiovascular and psychiatric morbidity, respectively (Dowman et al., 2011a).

#### 1.1.6.3.2 INSULIN SENSITISERS

Over the last decade, insulin-sensitising agents, including TZDs and metformin, have been employed in the majority of RCTs in NASH. Metformin, the first-line choice for poorly controlled type diabetes, has proven benefits on IR and liver enzymes (Angelico et al., 2007), however these did not translate to histological improvements (Musso et al., 2010). Even though US guidelines do not recommend metformin for the sole purpose of treating NASH (Chalasani et al., 2012), its potential to decrease the risk of HCC development warrants further investigation (Chen et al., 2013).

Well-designed, adequately powered RCTs of TZDs have consistently reported improvements in hepatic steatosis on liver biopsy (Belfort et al., 2006; Aithal et al., 2008; Ratziu et al., 2008; Ratziu et al., 2010b; Sanyal et al., 2010; Torres et al., 2011). There are, however, differences in histological efficacy between the types of TZD studied. Whereas rosiglitazone has failed to show an effect (Ratzu et al., 2008; Ratzu et al., 2010b), pioglitazone at doses of 30-40 mg/day has repeatedly been reported to improve inflammation and hepatocyte ballooning, and in some cases fibrosis (Belfort et al., 2006; Aithal et al., 2008; Sanyal et al., 2010; Torres et al., 2011). The largest RCT to be performed to date is the PIVENS study, which randomised 247 non-diabetic patients with biopsy proven NASH to pioglitazone (30 mg/day), the antioxidant vitamin E (800 IU/day), or placebo for 2 years (Sanyal et al., 2010). Pioglitazone resulted in significant histological improvements compared to placebo (47% vs. 21% resolved NASH), when NASH resolution was the outcome measure. This was despite the fact that a significant proportion of patients were excluded from the analysis, as they were found not to have NASH at baseline on retrospective central review (Sanyal et al., 2010).

It is important to note that all trials in TZDs have reported weight gains ranging from 1.5 to 4.7 Kg, which may impact on patient perception and uptake of the drug. Secondly, the majority of the trials including PIVENS were performed in patients without diabetes, thus their efficacy in the vast majority of patients with NASH (+ diabetes) remains unknown. Thirdly, there is still considerable debate regarding the long-term safety profile of TZDs in patients with NASH, who are already at increased risk of CVD morbidity and mortality. Due to the risks of such rosiglitazone has been removed from the market in Europe. In contrast, pioglitazone has been reported in a meta-analysis, consisting of 19 diabetes trials, to

significantly reduce myocardial infarction and death (Lincoff et al., 2007). There was, however, a higher rate of heart failure (Lincoff et al., 2007), and as a result of recent concerns raised with regards to bladder cancer (Lewis et al., 2011), pioglitazone has been discontinued in France and Germany (Cusi, 2012).

#### 1.1.6.3.3 ANTI-OXIDANTS

Prior to the PIVENS trial, the majority of RCTs of anti-oxidants (vitamin E, C and betaine) in NASH were small (*n*<20 on anti-oxidant in each trial) and had variable outcome measures/comparator arms (Harrison et al., 2003; Dufour et al., 2006; Nobili et al., 2008; Abdelmalek et al., 2009). PIVENS highlighted that high-dose vitamin E (800 IU/L) results in significant histological improvements in all components of NASH, with the exception of fibrosis (Sanyal et al., 2010). Even though this results are promising, as mentioned above they are not representative of patients with co-existing NASH and diabetes. Furthermore, although the absolute risk is small and inconsistent among studies, vitamin E (at doses >400 IU/L) may increase all-cause mortality (Armstrong et al., 2010).

#### 1.1.6.4 Future directions

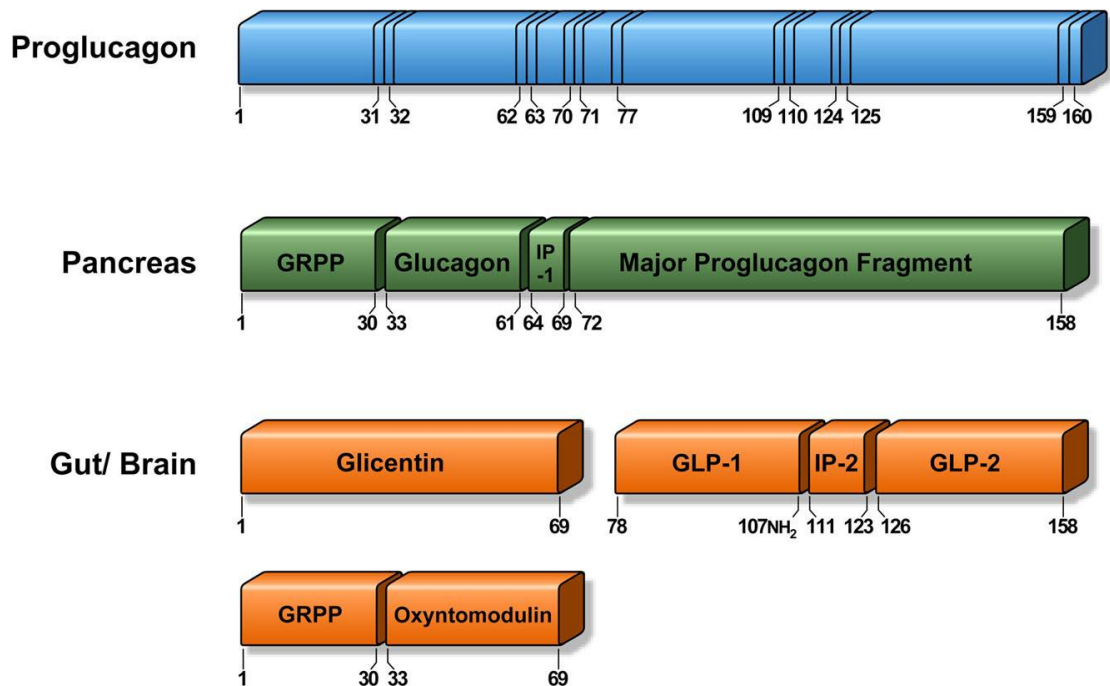
Even though the most recent findings with pioglitazone and vitamin E are promising, questions still remain with regards to their long-term safety and efficacy, especially in patients with co-existing type 2 diabetes and NASH. There is a pressing need to investigate novel therapies that not only target IR and lipotoxicity, but also reduce weight (fat mass) and

other risk factors of CVD. Due to their weight and glucose lowering properties, glucagon-like peptide 1 (GLP-1) based therapies represent a new therapeutic option in type 2 diabetes, but their impact on liver disease remains unknown.

## 1.2 Glucagon-like peptide-1 (GLP-1)

### 1.2.1 Physiology and actions of GLP-1

GLP-1 is a gut-derived peptide, and together with glucose-dependent insulintropic polypeptide (GIP), they form the two principal incretin hormones in the human body (Baggio and Drucker, 2007). The concept of the 'incretin (*intestinal secretion of insulin*) effect' was founded in the 1960's when scientists observed that oral glucose intake elicited a greater insulin response than intravenous glucose (Elrick et al., 1964). This led to the hypothesis that gut-derived factors play a key role in post-prandial insulin secretion. In the 1970s, GIP was isolated from the gut mucosa (Dupre et al., 1973; Brown et al., 1975) and a decade later GLP-1 was discovered after cloning and sequencing of mammalian proglucagon genes (Bell et al., 1983a; Bell et al., 1983b). In doing so it was noted that in addition to glucagon, two peptides largely homologous to glucagon were encoded and were subsequently named GLP-1 and GLP-2 (Holst, 2007) (Figure 1-3). GLP-2, however, had no effect on insulin release and was therefore not classified as an incretin hormone. In contrast, GLP-1 (a 30-31 amino acid peptide) is secreted from enteroendocrine L-cells, which are mainly located in the distal ileum and colon, in response to mixed meal ingestion or individual nutrients including carbohydrates, lipids (NEFA), amino acids and dietary fibres (Unger et al., 1968; Herrmann-Rinke et al., 1995). This in turn stimulates glucose-dependent insulin production and secretion via specific receptors on pancreatic islet  $\beta$ -cells, in keeping with the properties of an incretin hormone (Holst, 2007).



**Figure 1-3. GLP-1 is a cleavage product of proglucagon (Holst, 2007).**

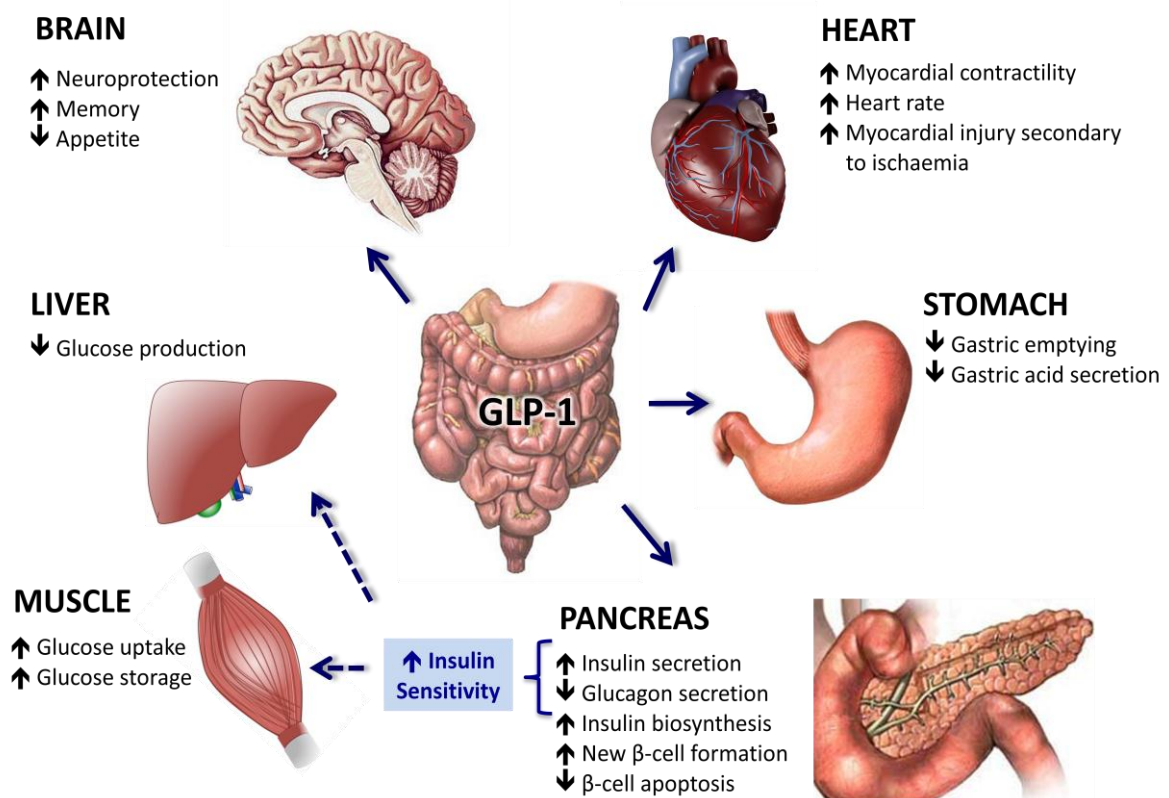
Proglucagon (160 amino acid sequence) is cleaved in the pancreas to form glicentin-related pancreatic polypeptide (GRPP), glucagon, intervening peptide-1 (IP-1) and the major proglucagon fragment. The latter is cleaved further in the intestinal mucosa to form the two glucagon-like peptides (GLP-1, GLP-2). The vertical lines represent the positions of basic amino acid residues at the typical cleavage sites.

The majority of circulating biologically active GLP-1 is found in the form of GLP-1(7-36) amide, with lesser amounts of the bioactive GLP-1(7-37) form also detectable (Kreymann et al., 1987; Baggio and Drucker, 2007). Both are thought, however, to be equally potent in their ability to stimulate insulin secretion in response to nutrient ingestion (Orskov et al., 1993). Even though GLP-1 secretion is meal related (blood concentrations = 10-30 pM), plasma levels are still detectable albeit in very low concentrations (5-10 pM) in the fasting state (Elliott et al., 1993). Basal rate secretion has mainly been demonstrated by the

lowering of fasting GLP-1 concentrations with exogenous somatostatin in human experiments (Toft-Nielsen et al., 1996).

GLP-1 maintains postprandial glucose homeostasis in humans via several modalities. In addition to its insulinotropic actions of pancreatic  $\beta$ -cells, it lowers circulating glucose by inhibiting glucagon release from pancreatic  $\alpha$ -cells and reducing hepatic glucose production. Importantly, all of these actions are glucose-dependent, therefore minimizing the risk of hypoglycaemia (Drucker, 2006). Indeed, the inhibitory effect of GLP-1 on glucagon has been shown to be absent at hypoglycaemic plasma glucose concentrations ( $\leq 3.7$  mmol/L) (Nauck et al., 2002). Even though the exact mechanism of GLP-1 on glucagon secretion remains unclear, it is likely mediated through the release of somatostatin (known to suppress glucagon) from pancreatic islet  $\alpha$ -cells (Orskov et al., 1988). GLP-1 has also been shown to increase  $\beta$ -cell mass, proliferation and reduce  $\beta$ -cell apoptosis in pre-clinical in vitro and rodent models (Brubaker and Drucker, 2004; Vilsbøll, 2009). In addition to its effect on pancreatic  $\alpha$ -cells and  $\beta$ -cell, GLP-1 has several other multiple tissue targets that collectively make it an attractive treatment option in type 2 diabetes and potentially other metabolic disorders (Figure 1-4). These include central appetite suppression (with reduced food intake) (Turton et al., 1996; De Silva et al., 2011), inhibition of gastric emptying (Nauck et al., 1997) and gastric acid secretion (resulting in lower postprandial glycaemia (Meier et al., 2003)), peripheral and central neuroprotection (Himeno et al., 2011; Gejl et al., 2012; Aviles-Olmos et al., 2013), and inhibition of osteoclastic bone resorption (Yamada et al., 2008). There is also growing evidence that GLP-1 has beneficial effects on cardiac function and myocardial injury (Nikolaidis et al., 2004; Read et al., 2012).





**Figure 1-4. Overview of the main actions of GLP-1 on various tissue types**

The multiple physiological actions of GLP-1 (*listed above*) are not necessarily representative of the native GLP-1 hormone (as is the case with pancreatic insulin secretion), but highlight the effects found in animal, in-vitro and human experiments with exogenous forms of GLP-1. GLP-1 also influences body temperature, fluid and salt retention and release of pituitary hormones (MacLusky et al., 2000; Gutzwiller et al., 2004). Figure adapted from (Drucker, 2006) and (Meier, 2012).

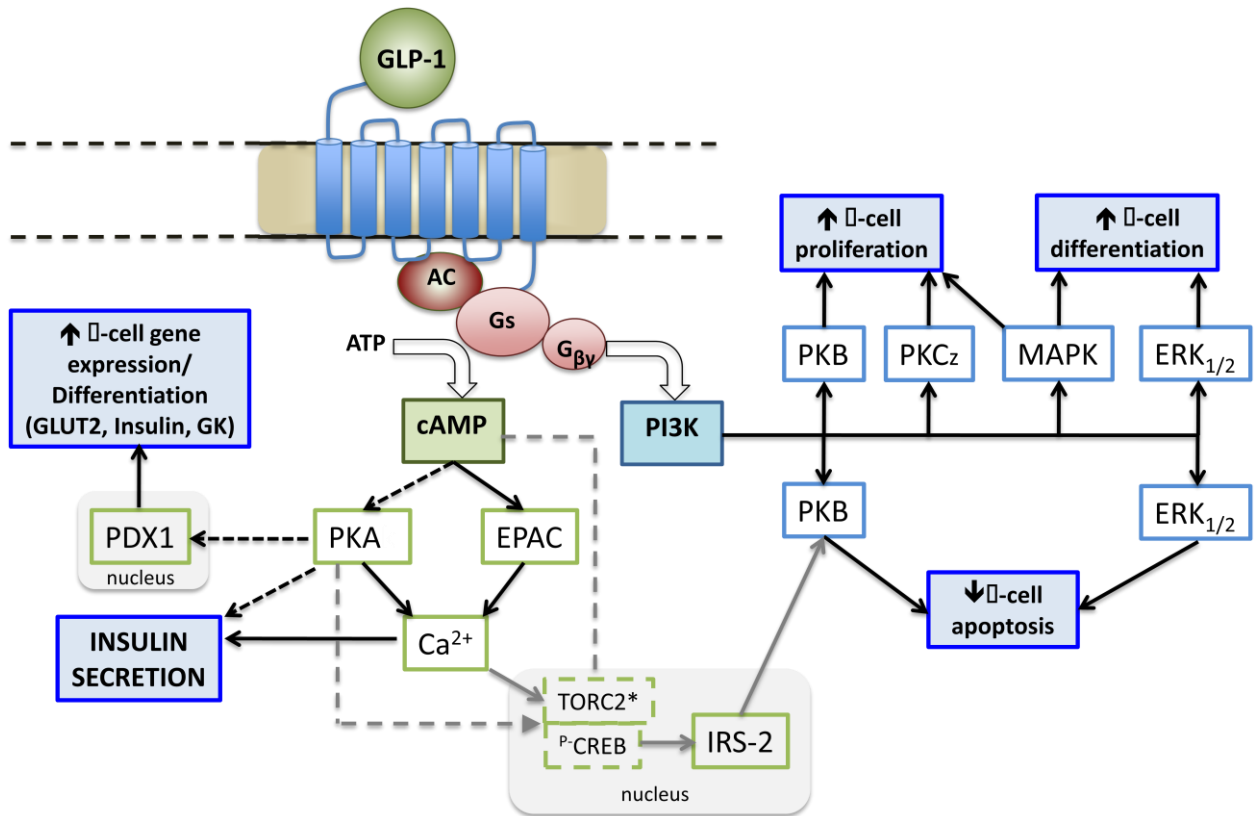
### 1.2.2 The GLP-1 receptor (GLP-1R)

The main physiological roles of GLP-1 are mediated by its specific receptor (GLP-1R), which is heterogeneously expressed in multiple organs including: pancreatic ducts and  $\beta$ -cells, heart, stomach (parietal cells), lungs, kidneys, intestine, pituitary, endothelium and the central

nervous system (hypothalamus) (Baggio and Drucker, 2007; Holst, 2007). Even though patterns of glycosylation may differ between GLP-1R sites (e.g. lung vs. islets), only a single GLP-1R has been identified to date (Wei and Mojsov, 1995). The function of the GLP-1R is not however known for many of these sites.

The GLP-1R is a trans-membrane G-protein-coupled receptor, which was originally isolated by Bernard Thorens in 1992 using expression cloning of a rat pancreatic islet cDNA library (Thorens, 1992). The same research group subsequently cloned the human receptor on pancreatic  $\beta$ -cells one year later (Thorens et al., 1993). The human GLP-1R gene spans 40kb, consists of at least 7 exons and has been mapped to chromosome 6 and band p21.1 (Baggio and Drucker, 2007). Binding of GLP-1 (or GLP-1R agonist) to the receptor causes activation (via stimulatory G-protein) of adenylate cyclase, which increases the intracellular concentration of cyclic adenosine monophosphate (cAMP). This in turn, triggers a variety of downstream signalling pathways in the various cell types (Holst, 2007).

A vast majority of our understanding of GLP-1 signalling pathways has been taken from experiments with pancreatic  $\beta$ -cells. In brief, increases in intracellular cAMP in  $\beta$ -cells lead to stimulation of insulin secretion (exocytosis) by two different pathways, which are either protein kinase A (PKA)-dependent or PKA-independent. In addition, the anti-apoptotic effects of GLP-1 on  $\beta$ -cells are mediated via the complementary signalling pathways of cAMP/PKA and phosphatidylinositol 3-kinase (PIK3) (Combettes, 2006; Holst, 2007). These pathways are described in more detail in Figure 1-5.



**Figure 1-5. GLP-1 signalling via G-protein coupled receptor activation in pancreatic  $\beta$ -cells.**

GLP-1 activation of the trans-membrane, G-protein (Gs) coupled GLP-1R stimulates adenylate cyclase (AC), which converts adenosine triphosphate (ATP) to cAMP. cAMP then activates protein kinase A (PKA) and the exchange protein directly activated by cAMP (EPAC). Activated PKA and EPAC trigger opening of  $\text{Ca}^{2+}$  channels and mobilisation of intracellular  $\text{Ca}^{2+}$  stores, respectively. The increased intracellular  $\text{Ca}^{2+}$  levels ultimately induce insulin release from the cell in response to glucose. In addition PKA activates pancreatic duodenal homeobox-1 (PDX-1), which is responsible for regulation of key  $\beta$ -cell gene expression (including insulin, GLUT2 and glucokinase (GK)). The anti-apoptotic effect of GLP-1 in pancreatic cells is via cAMP-dependent and phosphatidylinositol 3-kinase (PI3K) pathways. The former involves the response element binding protein (CREB) and its interaction with the co-activator TORC2 (transducer of regulated CREB activity), which results in the upregulation of the insulin receptor-2 (IRS-2) and subsequent protein kinase B (PKB) activation. The PI3K pathway is activated by GLP-1 via the G-protein dimer ( $\text{G}\beta\gamma$ ). This in turn triggers a cascade of downstream targets, including mitogen-activated protein kinases (MAPKs), extracellular-regulated kinases (ERKs), protein kinase C zeta ( $\text{PKC}\zeta$ ) and PKB (also known as AKT). Together these contribute to  $\beta$ -cell proliferation and differentiation, as well as decreasing  $\beta$ -cell apoptosis. GLP-1 dependent PKB activation also mediates its anti-apoptotic action via down regulating transcription factors FOXO1 and nuclear-factor- $\kappa\text{B}$  ( $\text{NF}\kappa\text{B}$ ). Figure is adapted from (Combettes, 2006; Holst, 2007).

Both exendin(9-39), a specific potent GLP-1R antagonist (Göke et al., 1993), and mice with a null mutation of GLP-1R (GLP-1R<sup>-/-</sup>) (Scrocchi et al., 1996) have been used to demonstrate the physiologic importance of GLP-1 ligand-receptor interactions. Exendin(9-39) has been shown to impair glucose tolerance and reduce glucose-stimulated insulin levels in both animal and human experiments (Schirra et al., 1998). Furthermore, the GLP-1R<sup>-/-</sup> mice have mild fasting hyperglycaemia and when challenged with oral or peripheral glucose demonstrate modest glucose intolerance and sub-optimal insulin secretion (Scrocchi et al., 1996). Both models of GLP-1 ligand-receptor interference also highlight the importance of the GLP-1R signaling in pancreatic  $\beta$ -cell development and survival (Li et al., 2003).

Several observations, however, have questioned whether there is more than one type of GLP-1R, through which GLP-1 exerts some of its physiological actions. For example, exendin(9-39) does not always inhibit the actions of GLP-1, as seen with gastrointestinal motility experiments in canines (Daniel et al., 2002). In addition, the cardioprotective effects of GLP-1 (e.g. reversing ischaemic injury) are reserved in GLP-1R<sup>-/-</sup> mice (Ban et al., 2008) and the non-insulinotropic GLP-1(9-36) amide (less active breakdown product of GLP-1(7-36)) has also been shown to enhance left ventricular function despite the presence of the GLP-1R inhibitor exendin(9-39) (Nikolaidis et al., 2005). The conflicting evidence with respect to the direct role of GLP-1R signaling in the adipose (Ruiz-Grande et al., 1992; Villanueva-Peñacarrillo et al., 2001), muscle (D'Alessio et al., 1994; Ryan et al., 1998) and liver, together with the controversy that surrounds the presence/absence of the GLP-1R in these tissues further supports the notion that GLP-1 may interact with other types of membrane

receptors (Baggio and Drucker, 2007). A detailed overview of the effects of GLP-1 on the liver is described below.

### **1.2.3 Development of GLP-1-based therapies for human use**

For decades, the mainstay of therapy for type 2 diabetes has focused on reducing hepatic glucose production (e.g. metformin), increasing insulin secretion from pancreatic  $\beta$ -cells (e.g. sulphonylurea) and reducing hepatic/peripheral IR (i.e. TZDs); all of which are key characteristics of type 2 diabetes. In recent years, however, incretin hormones have been the focus of considerable research activity in the treatment type 2 diabetes. This is largely due to the fact that the incretin effect is significantly reduced or absent in patients with type 2 diabetes (Nauck et al., 1986), which in turn contributes to impaired regulation of insulin and glucagon in these patients.

The majority of the focus has been on GLP-1 based therapies rather than GIP, due to the concordance of the physiological actions of GLP-1 with the therapeutic needs of patients with type 2 diabetes. In addition, even though secretion of GIP is maintained in these patients, its effect on pancreatic insulin secretion is markedly impaired (Baggio and Drucker, 2007; Nauck et al., 2011) and subsequently exogenous administration of GIP (even at supra-physiological concentrations) fails to restore appropriate insulin secretion in response to glucose (Nauck et al., 2011). In contrast, secretion of GLP-1 in response to meal ingestion is impaired in patients with type 2 diabetes and/or obesity, but its insulinotropic and glucagon-suppressive actions remain intact, which means continuous GLP-1 replacement is efficacious

(Nauck et al., 1993; Zander et al., 2002). Furthermore, as the insulinotropic effects of GLP-1 are glucose-dependent the risk of hypoglycaemia is very low. Lastly, as a result of the inhibitory effects of GLP-1 on appetite (centrally) and gastric emptying the potential for weight loss is advantageous over traditional anti-diabetes therapies, as they are largely associated with weight gain (i.e. TZDs, sulphonylureas).

Despite these promising physiological actions, the major drawback with native human GLP-1 as a clinically effective medical therapy is its short plasma half-life of 1-2 mins after intravenous and 5-10 mins after subcutaneous administration (Deacon et al., 1995). This is due to the rapid degradation of GLP-1 by the proteolytic enzyme, dipeptidyl peptidase-4 (DPP-4), which can be found in several tissue types including the kidney, lung, adrenal gland, spleen, intestine, liver, pancreas, CNS and several cell types, including macrophages. Most notably, it can be found on the luminal surface of endothelial cells lining the blood vessels that drain the intestinal mucosa, adjacent to the site of GLP-1 secretion (Hansen et al., 1999). DPP-4 metabolises the insulinotropic form of GLP-1(7-36) by cleaving off the two NH<sub>2</sub>-terminal amino acids to form the non-insulinotropic metabolites, namely GLP-1(9-37) or GLP-1(9-36) amide. After further enzyme degradation in the liver, only 10-15% of active GLP-1(7-36) reaches the systemic circulation (Holst, 2007).

From human studies it became clear that 24-hours infusion of native GLP-1 would be necessary to achieve satisfactory chronic glycaemic control (Hansen et al., 1999). However this is both expensive and clinically impractical in patients with type 2 diabetes. Two approaches have been taken by the pharmaceutical industry in order to circumvent this

limitation and maximise the potential of GLP-1 for clinical use. These include: 1) modifications of GLP-1 or human peptide analogues that avoid degradation of endogenous DPP-4 and renal clearance and 2) direct inhibition of the DPP-4 enzyme to prevent degradation of the active GLP-1 peptide.

By early 2013, three GLP-1R agonists had been approved in Europe and US for use in type 2 diabetes (bi-daily (BD) exenatide, once-weekly exenatide extended release (ER), and once-daily (OD) liraglutide) and with several others in development (Russell-Jones and Gough, 2012). As the GLP-1R agonists are protein-based they require subcutaneous administration, whereas DPP-4 inhibitors (known as 'gliptins') are orally administered. The latter, however, do not achieve the pharmacological plasma levels of GLP-1R agonists, as they only enhance the physiological levels of native endogenous GLP-1. This likely explains why they are weight neutral (vs. weight loss with GLP-1R agonists) and in head-to-head trials have inferior improvements in glycaemic control than GLP-1R agonists (Bergenstal et al., 2010; Pratley et al., 2011). DPP-4 inhibitors currently licensed in Europe and the US includes sitagliptin, saxagliptin, vildagliptin and linagliptin. A full description of the current and future GLP-1R agonists and DPP-4 inhibitors available for use in type 2 diabetes is beyond the scope of this review and are detailed elsewhere (Aroda et al., 2012; Cho et al., 2012; Russell-Jones and Gough, 2012).

#### 1.2.4 Exenatide (Byetta®; Amylin Pharmaceuticals)

Exenatide was the first GLP-1R agonist to be manufactured for human use. It is a synthetic version of exendin-4, a 39 amino acid peptide, which was originally isolated from the venom of the *Heloderma suspectum* lizard ('Gila monster'). Exendin-4 shares 53% amino acid sequence homology with native GLP-1 and is a potent agonist of the GLP-1R (Eng et al., 1992). Importantly, due to the fact that exendin-4 contains glycine at position 2 rather than arginine, it is resistant to DPP-4 metabolism (Baggio and Drucker, 2007). The resultant half-life (3-4 hrs) means that it can be given at a 5mg or 10mg dose BD via subcutaneous injection in order to achieve pharmaceutical levels (duration 4-8 hrs after single dose) that are 10-100-fold more potent than native GLP-1 (Cho et al., 2012). Between 2005-2006, exenatide was licensed in the US and Europe for the treatment of type diabetes in combination with one or more oral anti-hyperglycaemic therapy (Eli Lilly and Company Ltd, 2011b). Six years later the longer acting preparation of exenatide, known as exenatide ER (Bydureon®; Amylin Pharmaceuticals), was licensed. The sustained duration of drug delivery was achieved by incorporating microspheres of exenatide and poly-D-L-Lactic-co-glycolic acid to form a polymer that breaks down over time (Eli Lilly and Company Ltd, 2011a).

Prior to licensing an extensive clinical trials programme was performed for exenatide (10mg BD) and exenatide ER (2mg once-weekly) (Table 1-6). Over an average of 6 months, the higher dose of 10mg BD exenatide reduced HbA1c by 0.8-1.1% and weight by 1.6-2.8 kg (Buse et al., 2004; DeFronzo et al., 2005; Heine et al., 2005; Kendall et al., 2005; Nauck et al., 2007; Zinman et al., 2007), whereas 2mg once-weekly exenatide ER reduced HbA1c by 1.3-



1.9% and weight by 2.2-3.7 kg from baseline (Bergenstal et al., 2010; Buse et al., 2010a; Diamant et al., 2010; Blevins et al., 2011; Russell-Jones et al., 2012; Buse et al., 2013). In a head-to-head trial (DURATION-5), once-weekly exenatide ER showed significantly greater improvements in HbA1c and weight than BD exenatide (Blevins et al., 2011). Furthermore, exenatide has been shown to sustain improvements in glycaemic control and weight for up to 3 years in patients with type diabetes, thus implying that exenatide may prevent the progression of beta-cell failure (Riddle et al., 2006; Buse et al., 2007; Klonoff et al., 2008).

Additional post-hoc analysis has emerged over the last 2 years with regards to the beneficial effects of BD exenatide, and most exenatide ER, on other cardiovascular risk factors in patients with type 2 diabetes, including blood pressure and lipid profile (Grimm et al., 2013; Robinson et al., 2013; Wang et al., 2013a). The clinical extra-pancreatic effects of GLP-1R agonists will be discussed in more detail below.

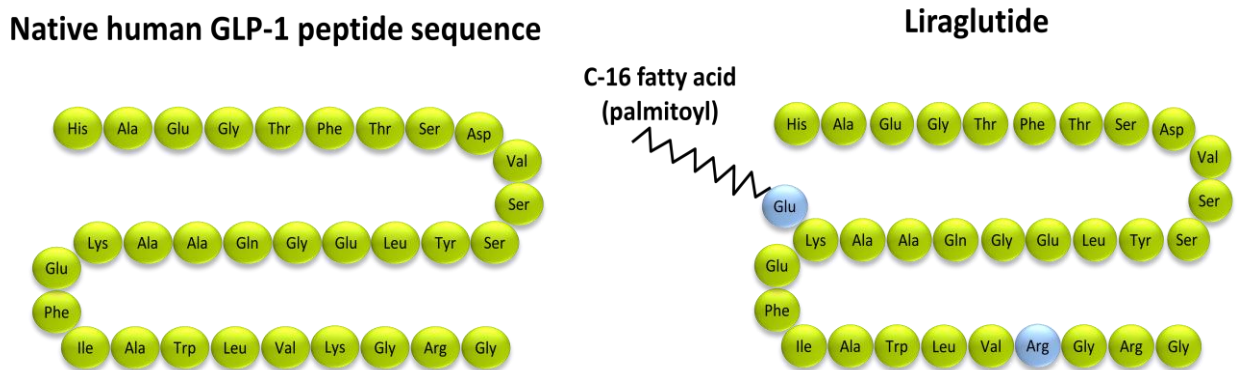
<b>Trial name (if available) Author (order of year)</b>	<b>Study Size</b>	<b>Study Duration</b>	<b>Concurrent Treatment</b>	<b>Comparators</b>	<b>HbA1c (%) change from baseline</b>	<b>Weight (Kg) change from baseline</b>
(Buse et al., 2004)	377	30 weeks	SU	10 µg exenatide BD Placebo	-0.86 (0.11)*** +0.12 (0.09)	-1.6 kg (0.3)* -0.6 kg (0.3)
(DeFronzo et al., 2005)	336	30 weeks	MET	10 µg exenatide BD Placebo	-0.78 (0.10)*** +0.08 (0.10)	-2.8 kg (0.5)*** -0.3 kg (0.3)
(Kendall et al., 2005)	733	30 weeks	MET + SU	10 µg exenatide BD Placebo	-0.77 (0.10)*** +0.23 (0.10)	-1.6 kg (0.2)* -0.9 kg (0.2)
(Heine et al., 2005)	551	26 weeks	MET + SU	10 µg exenatide BD Insulin glargine OD	-1.11% -1.11%	-2.3 kg <sup>&amp;&amp;</sup> +1.8 kg
(Zinman et al., 2007)	233	16 weeks	TZD ± MET	10 µg exenatide BD Placebo	-0.89 (0.09)*** +0.09 (0.1)	-1.75 (0.25)*** -0.24 (0.26)
(Nauck et al., 2007)	501	52 weeks	MET + SU	10 µg exenatide BD Insulin aspart BD	-1.04 (0.07) -0.89 (0.06)	-2.5 kg (0.2) <sup>&amp;&amp;&amp;</sup> +2.9 kg (0.2)
DURATION-1 (Buse et al., 2010a)	258	30 weeks	MET/SU/TZD	2 mg exenatide ER 10 µg exenatide BD	-1.9 (0.1)* -1.5 (0.1)	-3.7 kg (0.5) -3.6 kg (0.5)
DURATION-2 (Bergenstal et al., 2010)	491	26 weeks	MET Placebo (oral/sc)	2 mg exenatide ER 45 mg TZD 100 mg sitagliptin	-1.5% <sup>&amp;</sup> -1.2% -0.9%	-2.3 kg <sup>&amp;&amp;&amp;</sup> +2.8 kg -0.8 kg
DURATION-3 (Diamant et al., 2010)	456	26 weeks	MET± SU	2 mg exenatide ER Insulin glargine OD	-1.5 (0.05) <sup>&amp;</sup> -1.3 (0.06)	-2.6 kg (0.2) <sup>&amp;&amp;&amp;</sup> +1.4 kg (0.2)
DURATION-4 (Russell-Jones et al., 2012)	820	26 weeks	None	2 mg exenatide ER 45 mg TZD 100 mg sitagliptin OD 2.5 g MET	-1.53 (0.07) <sup>+++</sup> -1.63 (0.08) -1.15 (0.08) -1.48 (0.07)	-2.0 (0.2) <sup>+++</sup> +1.5 (0.3) -0.8 (0.3) -2.0 (0.2)
DURATION-5 (Blevins et al., 2011)	252	24 weeks	MET/SU/TZD	2 mg exenatide ER 10 µg exenatide BID	-1.6 (0.1) <sup>&amp;&amp;&amp;</sup> -0.9 (0.1)	-2.3 (0.4) <sup>&amp;</sup> -1.4 (0.4)
DURATION-6 (Buse et al., 2013)	912	26 weeks	MET/SU/TZD	2 mg exenatide ER 1.8 mg liraglutide OD	-1.28 (0.05) -1.48 (0.05)	-2.7 (0.2) -3.6 (0.2)

**Table 1-6. Summary of clinical trials (>16 duration) of exenatide and long-acting exenatide ER.**

In all trials listed HbA1c was the primary end-point. 10mg BD Exenatide/2mg once-weekly Exenatide ER was either compared to an injectable placebo (\*p<0.05; \*\*<0.01; \*\*\*<0.001) or an active comparator (&p<0.05; &&<0.01; &&&<0.001; +++<0.001 vs. sitagliptin only). Key: BD, twice daily; ER, extended release; HbA<sub>1c</sub>, glycated haemoglobin; MET, metformin; OD, once daily; SU, sulphonylurea; TZD, thiazolidinedione. All values are means±SE (when available).

### 1.2.5 Liraglutide (Victoza®; Novo Nordisk A/S)

Liraglutide is a long-acting human GLP-1 analogue that shares 97% sequence homology to native GLP-1. Liraglutide differs from native GLP-1 by the addition of a C16 fatty acid chain at lysine<sup>26</sup> in the peptide and the substitution of lysine<sup>34</sup> with arginine<sup>34</sup>, which in turn improve the binding of albumin, inhibit renal clearance and importantly make the peptide less accessible to metabolism by DPP-4 (Knudsen et al., 2000) (Figure 1-6).



**Figure 1-6. Liraglutide shares 97% amino acid sequence homology to native GLP-1.**

Two modifications to the amino acid sequence of GLP-1 are made, namely a Glu-tagged fatty acid is acetylated to lysine at position 26, and the lysine at position 34 is replaced with arginine (*highlighted in blue*). Therefore, the problem of the short half-life, which is the major clinical drawback of native GLP-1, is overcome with liraglutide (Knudsen et al., 2000).

In comparison to exenatide (peak plasma concentration at 2 hrs), liraglutide is slowly absorbed from the injection site (peak at 9-12 hrs) and the resultant extended half-life of 11-13 hours makes it suitable for once daily administration via the subcutaneous route (Knudsen et al., 2000).

#### *1.2.5.1 Liraglutide - Pre-clinical data*

Pre-clinical receptor studies have shown that, despite modifications to the native GLP-1 peptide, liraglutide retains selectivity, potency and affinity for the cloned human GLP-1 receptor (Knudsen et al., 2000). In rodent models of obesity and diabetes, liraglutide has been shown to lower blood glucose, stimulate insulin secretion, decrease plasma glucagon levels, inhibit gastric emptying, inhibit food intake, decrease body weight, improve  $\beta$ -cell function and  $\beta$ -cell mass when administered subcutaneously (Larsen et al., 2001; Rolin et al., 2002; Sturis et al., 2003; Knudsen, 2010).

#### *1.2.5.2 Liraglutide – Effect on glycaemic control*

In accordance with the rodent experiments, mode-of-action human trials demonstrated glucose lowering (fasting plasma glucose, postprandial glucose), increased insulin secretion, restored  $\beta$ -cell responsiveness to increasing glucose concentrations and delayed gastric emptying after a single subcutaneous dose of liraglutide. Subsequently, improvements in HbA1c were consistent throughout the early phase clinical trials of liraglutide (Chia and Egan, 2008; Verspohl, 2009). Importantly, during low blood glucose levels liraglutide did not impair glucagon action or the general counter-regulatory response, indicating a low risk of hypoglycaemia (Madsbad et al., 2004). Subsequently, a comprehensive phase III evaluation consisting of six large randomised-controlled trials of liraglutide in patients with poorly controlled type diabetes was performed. The ‘Liraglutide Effect and Action Diabetes (LEAD) program’ involved 6500 participants from 41 countries world-wide and in total 4445 patients

received liraglutide (Buse et al., 2009; Garber et al., 2009; Marre et al., 2009; Nauck et al., 2009; Russell-Jones et al., 2009; Zinman et al., 2009) (Table 1-7). As a result of findings in the LEAD program, 1.2mg liraglutide OD was licensed for use in poorly-controlled type diabetes in Europe in 2009 and a year later by the FDA in the US (Novo Nordisk A/S, 2009).

In summary, the LEAD program highlighted that 26-52 weeks treatment with either 1.2mg or 1.8mg liraglutide OD results in significant reductions in HbA1c of 0.9-1.5% and 1.0-1.5%, respectively (Table 1-7). Most notably, in a direct head-to-head trial (LEAD-6) 26-weeks treatment 1.8mg liraglutide OD significantly reduced HbA1c and fasting plasma glucose more than 10mg exenatide BD, with no additional risk of hypoglycaemia (Buse et al., 2009). Furthermore, the 16-week cross-over extension of LEAD-6 highlighted switching from exenatide to liraglutide resulted in an additional 0.3% reduction in HbA1c (Buse et al., 2010b), again without increasing adverse events (*discussed in detail below*). More recently in the DURATION-6 trial, the same authors reported greater glycaemic control (albeit no significant difference) with 1.8mg liraglutide compared with the once-weekly preparation of exenatide (Buse et al., 2013).

<b>Trial name Author (order of year)</b>	<b>Study Size</b>	<b>Study Duration</b>	<b>Concurrent Treatment</b>	<b>Comparators</b>	<b>HbA<sub>1c</sub> (%) change from baseline</b>	<b>Weight (Kg) change from baseline</b>
LEAD-1 (Marre et al., 2009)	1018	26 weeks	SU	1.8 mg liraglutide OD 1.2 mg liraglutide OD 4 mg rosiglitazone Placebo	-1.13***, &&& -1.08***, &&& -0.44 +0.23	-0.2&&& +0.3&&& +2.1 -0.1
LEAD-2 (Nauck et al., 2009)	1091	26 weeks	MET	1.8 mg liraglutide OD 1.2 mg liraglutide OD 4 mg glimepiride Placebo	-1.00*** -0.97*** -0.98 +0.09	-2.8 (0.2)** , &&& -2.6 (0.2)** , &&& +1.0 (0.2) -1.5 (0.3)
LEAD-3 (Garber et al., 2009; Garber et al., 2011)	746	52 (+52 ext.) weeks	None	1.8 mg liraglutide OD 1.2 mg liraglutide OD 8 mg glimepiride	-1.1&& -0.9& -0.6	-2.7&&& -2.1&&& +1.1 kg
LEAD-4 (Zinman et al., 2009)	533	26 weeks	MET +TZD	1.8 mg liraglutide OD 1.2 mg liraglutide OD Placebo	-1.5 (0.1)*** -1.5 (0.1)*** -0.5 (0.1)	-2.0 (0.3)*** -1.0 (0.3)*** +0.6(0.3)
LEAD-5 (Russell-Jones et al., 2009)	581	26 weeks	MET + SU	1.8 mg liraglutide OD Insulin glargine Placebo	-1.33 (0.09)*, & -1.09 (0.09) -0.24 (0.11)	-1.8 (0.3)&&& +1.6 (0.3) -0.4 (0.4)
LEAD-6 (Buse et al., 2009)	464	26 weeks	MET± SU	1.8 mg liraglutide OD 10 µg exenatide BD	-1.12 (0.08) &&& -0.79 (0.08)	-3.24 (0.33) -2.87 (0.33)
LIRA-DPP4-1860 (Pratley et al., 2011)	665	52 weeks	MET	1.8 mg liraglutide OD 1.2 mg liraglutide OD 100 mg sitagliptin OD	-1.51&&& -1.29&&& -0.88	-3.68&&& -2.78&&& -1.16

**Table 1-7. Summary of 7 phase III clinical trials (LEAD program + 1860 trial) investigating the effect of liraglutide on glycaemic control in type 2 diabetes.**

In all trials listed HbA<sub>1c</sub> was the primary end-point. 1.2mg or 1.8mg liraglutide OD was either compared to an injectable placebo (\*p<0.05; \*\*<0.01; \*\*\*<0.001) or an active comparator (&p<0.05; &&<0.01; &&&<0.00). Key: BD, twice daily; HbA<sub>1c</sub>, glycosylated haemoglobin; MET, metformin; OD, once daily; SU, sulphonylurea; TZD, thiazolidinedione. All values are means±SE (when available).

#### *1.2.5.3 Liraglutide – Effect on weight*

As demonstrated in the LEAD program in patients with type 2 diabetes, liraglutide is also a promising therapy for obesity, with reported weight loss ranging from 0.2-3.2 kg with 1.8mg

over 6 months (Table 1-7). Of note, weight loss was maximal during the first 16-weeks of therapy, but remained stable for over one year in LEAD-3 trial (Garber et al., 2009). Using Dual-energy X-ray absorptiometry (DEXA) and CT imaging, a single study highlighted that weight loss occurs predominantly in abdominal fat (visceral > abdominal subcutaneous) with liraglutide (Jendle et al., 2009). In direct comparison to exenatide and exenatide ER, greater weight loss was seen with 1.8mg liraglutide in patients with type 2 diabetes; albeit the difference was only significant when compared to the longer-acting preparation (-3.58 vs. -2.68 kg;  $p<0.05$ ) (Blevins et al., 2011).

Liraglutide's weight inducing effect has also been described in a cohort of 564 non-diabetic obese subjects, in comparison to the approved weight-loss agent orlistat, a well-known gastrointestinal lipase inhibitor (Astrup et al., 2009). 20-weeks treatment with liraglutide (1.8, 3.0 mg) significantly reduced weight (-5.5 kg, -7.2 Kg, respectively) in comparison to orlistat (-4.1 kg) and placebo (-2.8 Kg) ( $p<0.005$ ). Unlike liraglutide's glucose-lowering effects, weight loss appears to be dose dependent. The 2-year extension of this trial revealed that weight loss was sustainable and that the prevalence of pre-diabetes (impaired glucose tolerance [IGT] and/or IFG) and metabolic syndrome (ATP III classification) decreased by over 50% with doses between 1.8mg and 3.0mg (Astrup et al., 2012).

#### *1.2.5.4 Liraglutide – Effect on blood pressure, lipids and other CVD measures*

In keeping with data pooled from exenatide trials (Okerson et al., 2010), meta-analysis of the LEAD program highlighted that liraglutide can reduce systolic blood pressure by an average

of 2.5 mmHg (range 2-7 mmHg) over 6 months (Robinson et al., 2013). In contrast, no significant differences in diastolic blood pressure were seen in the LEAD program. Similarly to changes in weight, the largest reductions in systolic blood pressure (averaging -11 mmHg change) were seen in individuals who had abnormal readings at baseline (140-190 mmHg). Of interest, reductions in systolic blood pressure occurred in the first two weeks, prior to significant weight loss, thus suggesting that the anti-hypertensive effect of GLP-1 might be weight-independent. Even though the mechanism remains poorly understood, plausible explanations include the natriuretic and vasodilatory effects of GLP-1 agonists, which have been previously described in both salt-sensitive rodent and non-primate models (Edwards et al., 1997; Liu et al., 2010).

There have been conflicting reports with regards to the effects of exenatide on serum lipids (Kendall et al., 2005; Blonde et al., 2006), whilst a recent large post-hoc analysis of 6 months exenatide ER has shown modest reductions in fasting total cholesterol (-0.17 mmol/L) (Grimm et al., 2013). There is a paucity of well-designed studies investigating the effects of liraglutide on fasting and postprandial dyslipidaemia; the latter of which has been shown to be an independent risk factor for CVD (Nordestgaard et al., 2007). However, in the last few months a placebo-controlled cross-over trial (20 patients) has reported that 21 days treatment with 1.8mg Liraglutide treatment significantly reduces excursions of triglyceride after a fat-rich meal. Furthermore, the authors comment that this effect appears to be independent of delayed gastric emptying, which was robustly assessed with paracetamol absorption and the <sup>13</sup>C-octanoate breath tests (Hermansen et al., 2013). The effects of GLP-1 on post-prandial dyslipidaemia, might in part, contribute to the improvements in



endothelial function that have been reported with intravenous GLP administration and exenatide (Koska, 2012; Noyan-Ashraf et al., 2013).

In a recent study, weight neutral doses of liraglutide (7 days duration only) were shown to activate several cardioprotective pathways (including insulin sensitisation, reversal of TNF $\alpha$  expression, cardiac endoplasmic reticulum stress responses and also improve measures of cardiac function) in mice on high-fat diets (Noyan-Ashraf et al., 2013). Although a meta-analysis of the LEAD program (including data on exenatide) has shown baseline improvements in non-invasive serum markers of CVD risk (namely brain natriuretic peptide and high-sensitivity CRP) with 1.8mg liraglutide (Unger and Parkin, 2011), this was not reproducible in patients without diabetes (Astrup et al., 2009). At present, it remains uncertain whether use of GLP-1 agonists will have a substantial impact on hard cardiovascular end-points (i.e. myocardial infarction, cardiac death), but the effects reported in-vitro (Bose et al., 2005) and with experimental in-vivo models of GLP-1 infusions are promising (Nikolaidis et al., 2004; Nikolaidis et al., 2005; Sokos et al., 2007; Noyan-Ashraf et al., 2013).

#### *1.2.5.5 Liraglutide – insulin resistance (IR)*

There is a vast amount of literature, dating back to the 1990's, on the effects of native GLP-1 and GLP-1R agonists effect on  $\beta$ -cell function and insulin secretion, using the well-validated hyperinsulinaemic hyperglycaemic clamp technique. Data regarding insulin sensitivity, however, is less clear and most reports have been inconsistent until recently (Nielsen et al.,

2004a). This is likely due to the large heterogeneity between studies with regards to study population (i.e. healthy volunteers, elderly, type 2 diabetes, type 1 diabetes, in the presence or absence of obesity), role of GLP-1 administration (peripheral/central vein vs. subcutaneous injection) and the modality for assessing insulin sensitivity (i.e. homeostatic model assessment of IR [HOMA-IR], oral glucose tolerance test [OGTT] insulin-glucose ratio, and various euglycaemic clamp techniques). The most robust human studies have incorporated various hyperinsulinaemic euglycaemic clamp techniques to evaluate the effect of GLP-1 on endogenous glucose production (i.e. hepatic insulin sensitivity) and glucose disposal in the muscle (muscle insulin sensitivity). In brief, studies have shown that short infusions (single infusions) of native GLP-1 or GLP-1R agonists (up to 6 weeks duration) improve hepatic insulin sensitivity in healthy volunteers (D'Alessio et al., 1994; Prigeon et al., 2003), patients with type 2 diabetes (Gutniak et al., 1992; Zander et al., 2002) and in the elderly (Gutniak et al., 1992; Meneilly et al., 2001; Zander et al., 2002). There are, however, discrepancies with regards to GLP-1 effect on muscle insulin sensitivity (D'Alessio et al., 1994; Orskov et al., 1996; Vella et al., 2002; Prigeon et al., 2003) and whether or not the actions of GLP-1 are independent of pancreatic hormone secretion (Meneilly et al., 2001; Prigeon et al., 2003; Seghieri et al., 2013). Again, these discrepancies are likely due the small sample sizes (7-14 patients maximum), the presence/absence of diabetes in the study populations and importantly, whether or not the secretion of islet hormones was controlled for (i.e. use of a somatostatin analogue). There are no human studies that have assessed the effect of GLP-1 on adipose insulin resistance.

Despite a thorough literature review, the only studies that have assessed the effect of liraglutide (or NN2211 derivative) on human insulin sensitivity have used simple markers, rather than euglycaemic clamp techniques. HOMA-IR has been studied as a secondary measure of efficacy in several clinical trials of liraglutide. Even though studies have consistently reported improvements in markers of  $\beta$ -cell function with liraglutide (proinsulin:insulin ratio, HOMA-B) (Davies et al., 2011; Seghieri et al., 2013), results with HOMA-IR have been inconsistent. In LEAD-3, both 1.2 mg and 1.8 mg liraglutide monotherapy improved HOMA-IR in comparison to the sulphonylurea, glimepiride (1.2 mg: -0.65%,  $p < 0.05$ ; 1.8 mg: -1.35%,  $p < 0.01$  vs. glimepiride) (Garber et al., 2009). Similarly, HOMA-IR significantly improved with 1 years treatment with 1.8mg liraglutide versus the DPP-4 inhibitor, sitagliptin (-1.36% vs. -0.41,  $p = 0.04$ ) (Pratley et al., 2011). However, in the other 4 major phase III trials liraglutide did not significantly improve HOMA-IR when compared to placebo (Marre et al., 2009; Nauck et al., 2009; Russell-Jones et al., 2009; Zinman et al., 2009). Whether this truly reflects a lack of effect of the GLP-1R agonist on insulin resistance or not is unclear. This might, however, represent a limitation of HOMA-IR ( $\text{Fasting glucose} \times \text{Fasting insulin} / 22.5$ ) as a serial marker of drug-induced changes of insulin sensitivity, especially if the drugs are insulinotropic (Wallace et al., 2004).

#### *1.2.5.6 Liraglutide – Safety and tolerability*

In general, liraglutide is well-tolerated and its safety profile is in accordance with observations from administration of both native GLP-1 and exenatide (Novo Nordisk A/S, 2009).

#### 1.2.5.6.1 GASTROINTESTINAL ADVERSE EVENTS (AE)

Nausea (10-40% of patients), diarrhoea and vomiting (5-10%) were the most frequently reported events during the phase III LEAD program (Blonde and Russell-Jones, 2009). These symptoms appeared to be dependent on various factors, including portion size, meal frequency and potentially BMI (Meier, 2012). In general, the gastrointestinal AEs are transient, and in the vast majority subside after 4-8 weeks of treatment, which is thought to be as a result gastrointestinal tolerability. Dose titration schemes have been recommended for the different types of GLP-1R agonists in order to mitigate these symptoms in the first few weeks. The frequency of nausea is similar amongst all three approved GLP-1R agonists, but it appears less persistent with 1.8mg liraglutide OD than with 10mg exenatide (i.e. by week 6 rates of nausea were 8% vs. 16%, respectively (Buse et al., 2009)). Interestingly, in most of the studies with liraglutide (Blonde and Russell-Jones, 2009) and exenatide (Klonoff et al., 2008) the beneficial effects of weight loss appeared to be independent of these gastrointestinal side effects, with similar degrees of weight loss occurring in patients with and without nausea and vomiting.

#### 1.2.5.6.2 HYPOGLYCAEMIA

Due to the glucose-dependent actions of liraglutide few minor (i.e. symptomatic or plasma glucose  $<3.1$  mmol/L) and only 7 major episodes of hypoglycaemia (i.e. requiring third-part assistance) have been reported across the phase III trials of liraglutide (Blonde and Russell-Jones, 2009). The frequency of minor hypoglycaemic AEs was greater when liraglutide was

used in conjunction with a sulphonylurea (8-27%) than not (3-12%) (Gough, 2012). This is likely due to the synergistic effect on pancreatic insulin secretion, but also the fact that sulphonylurea actions are not glucose-dependent. Indeed, 6/7 major hypoglycaemic attacks were observed with sulphonylurea combination therapy, and only 1/7 reported with liraglutide with metformin, in which the blood glucose concentration was recorded at 3.6 mmol/L. No acute medical assistance was required in any of the events (Blonde and Russell-Jones, 2009). Similarly to the gastrointestinal AEs, despite the greater efficacy in glycaemic control the rate of hypoglycaemia was lower with liraglutide in the head-to-head trial with exenatide (1.9 vs. 2.6 events per patient year;  $p < 0.05$ ) (Buse et al., 2009). Of note, there were no confirmed cases of hypoglycaemia in non-diabetic patients receiving liraglutide at doses ranging from 1.2-3.0 mg (Astrup et al., 2009; Astrup et al., 2012).

#### 1.2.5.6.3 IMMUNOGENICITY

As a result of its 97% sequence homology to native GLP-1, the proportion of patients developing antibodies against liraglutide in the LEAD program was low (3-10%) (Buse et al., 2011). In contrast as many as 40-60% of patients receiving exenatide have antibody formation (DeFronzo et al., 2005; Buse et al., 2011). Even though there is no evidence to suggest that antibody formation against liraglutide compromises clinical efficacy or safety, data from the LEAD-6 trial indicates that exenatide antibody formation correlates with smaller reductions in HbA1c (Buse et al., 2009; Buse et al., 2011).

#### 1.2.5.6.4 PANCREATITIS

Cases of pancreatitis have been reported in animals (Nachnani et al., 2010; Gier et al., 2012), and human reports (Denker and Dimarco, 2006; Ahmad and Swann, 2008) with GLP-1R agonists and DPP-4 inhibitors. Therefore concerns have been raised over a potential relationship between these therapies and acute/chronic pancreatitis, yet experts remain divided with regards to whether there is a true causal relationship (Butler et al., 2013b; Nauck, 2013). Results regarding pancreatic mass, histology and enzymes from animal studies (mainly rodent) are variable depending on the type of incretin mimetic used whereby positive findings with exenatide (Nachnani et al., 2010; Gier et al., 2012) have not been reproduced with liraglutide (Nyborg et al., 2012; Vrang et al., 2012).

As of June 2013, 8 cases (from pool of 6628 patients receiving drug) of pancreatitis have been reported in patients receiving liraglutide in clinical trials, versus only one case in the comparator arms (from a pool of 1877 patients). This amounts to incidence rates of 1.6 cases/1000 patient-years of exposure with liraglutide in comparison to 0.6 cases/1000 patient-years with other anti-diabetes therapy ( $p=0.6948$ ) (personal communication from Novo Nordisk). These rates are well within the predicted range of a population of type 2 diabetics (0.5-5.6 cases/1000 patient-years of exposure), who have approximately double the risk of healthy individuals (Noel et al., 2009; Garg et al., 2010; Gonzalez-Perez et al., 2010). In addition, 2 cases of pancreatic carcinoma have been reported from the LEAD program (63 year-old female 22 weeks on treatment; 50 year old male 1 week on treatment)

in patients receiving liraglutide. Both of which were judged not be related to liraglutide by an independent panel of experts (Buse et al., 2009; Pratley et al., 2011).

Given the small numbers of cases reported in the LEAD program a causal relationship between liraglutide and pancreatic disease can neither be established nor excluded at present. However, in light of conflicting systematic reviews (including FDA databases, industrial reviews, medical insurance reviews) and meta-analyses that exist in the literature (Garg et al., 2010; Elashoff et al., 2011; Alves et al., 2012; Singh et al., 2013), which is likely due to discrepancies in diagnostic criteria, the current expert advice is to avoid use in patients with a history of pancreatic disease and stop treatment when the onset of pancreatitis is suspected.

#### 1.2.5.6.5 THYROID (MEDULLARY) C-CELL CARCINOMA

Medullary thyroid carcinoma (MTC) is an extremely rare form of thyroid cancer, with <600 cases/year in the US (Parks and Rosebraugh, 2010). During the extensive pre-clinical development of liraglutide, a dose-dependent increase in thyroid C-cell hyperplasia and calcitonin release (a marker of MTC in humans) was seen in rodents (Knudsen, 2010). Furthermore, liraglutide administration was associated with MTC development in rats. However, these findings have not be replicated in non-human primates despite exposure >60 times that seen in humans.

During the LEAD trials approximately 2% of patients showed increases blood calcitonin levels ( $\geq 20$  ng/L), irrespective of whether they were treated with liraglutide or not. Subsequently, seven cases of C-cell hyperplasia were detected (5 liraglutide: 2 comparator) and all underwent thyroidectomies without complications (personal communication from Novo Nordisk A/S). Despite the fact that the risk of developing MTC secondary to liraglutide is very low, the fact that the human relevance of the previous findings in rodents remains unclear necessary cautions should be taken (Novo Nordisk A/S, 2009). On licensing the product in 2010, the US FDA placed a 'black box' warning about MTC and liraglutide is currently contraindicated in patients with a personal or family history of MTC and multiple endocrine neoplasia (MEN) type 2 syndrome (Parks and Rosebraugh, 2010). Liraglutide is not contraindicated in patients with a history of over or underactive thyroid disease.

Calcitonin screening for MTC has a high rate of false positives (e.g. inflammation) and has a low positive predictive value (PPV<10% for basal levels <100ng/L). Despite this, the EU Summary of Product Characteristics (SmPc) still recommended that clinical trials involving GLP-1R agonists should monitor calcitonin levels and thyroid abnormalities closely (Novo Nordisk A/S, 2009).

#### 1.2.5.6.6 LIVER DISEASE/INJURY

Studies to date have highlighted that the pharmacokinetics of liraglutide are not compromised by hepatic impairment (Verspohl, 2009). However, due to the limited therapeutic experience with liraglutide in patients with known active/chronic liver disease,



European guidelines do not recommend liraglutide therapy in this patient population. However, the American guidelines recommend cautious use with no dose alterations in patients with known liver disease (Novo Nordisk A/S, 2009). Until further data is available, this could have huge implications for patients who type 2 diabetes and associated NASH.

### **1.2.6 Potential for GLP-1 based therapies in NAFLD**

Over the last decade, there has been an expansion of pre-clinical data highlighting that GLP-1 based therapies have the potential, via direct and indirect mechanisms, to reverse hepatic steatosis and injury in the metabolic-diseased state.

#### *1.2.6.1 Pre-clinical data (in-vitro/in-vivo studies)*

##### **1.2.6.1.1 ANTI-STEATOTIC EFFECT OF GLP-1 IN ANIMAL MODELS OF NAFLD**

Several animal models of hepatic steatosis have been used to study the effects of chronic GLP-1 administration on the liver and associated metabolic features (Ding et al., 2006; Samson et al., 2008; Ben-Shlomo et al., 2011; Samson et al., 2011; Sharma et al., 2011; Shirakawa et al., 2011; Tomas et al., 2011a; Lee et al., 2012; Mells et al., 2012; Trevaskis et al., 2012). These include obese *Ob/Ob* leptin deficient mice (or leptin receptor deficient, *Db/Db*), wild-type (C57BL/6) mice fed a variety of high fat diets (HFD, +/- fructose) and DPP-4 deficient rats on HFD. Even though a wide range of techniques have been used to achieve

supra-physiological levels of GLP-1 in these rodent models, all studies consistency found that GLP-1 reduces hepatic lipid accumulation in-vivo (Table 1-8).

In 2006, Ding and colleagues were the first to convincingly show that exendin-4 decreases hepatic triglyceride content and liver enzymes in a genetic (*leptin-deficient*) murine model of obesity (Ding et al., 2006). Trevaskis et al recently validated these findings in the same mouse model on fed a high fat diet, rather than chow alone. In addition, they found that despite a short-duration of therapy (4-weeks), albeit via a continuous pump, there was evidence to suggest that liver fibrosis had regressed on histological assessment and collagen-1 immunostaining (marker of fibrogenesis) with GLP-1 (Trevaskis et al., 2012). A valid limitation of both of these mouse experiments is the fact that leptin deficiency may have confounded the metabolic changes seen with GLP-1, as leptin and GLP-1 are known to interact with regards to appetite (Williams et al., 2006).

Author, year (in year order)	Treatment used	Mode/duration of drug delivery	Animal model	Hepatic findings	Weight loss
(Ding et al., 2006)	Exendin-4	IP injections; 8.5 wks	Ob/Ob mice + normal chow	↓ Hepatic steatosis ↓ ALT ↓ Oxidative stress ↓ Insulin resistance (HOMA-IR)	Yes
(Samson et al., 2008)	Exendin-4	Gene therapy (HDAd-Ex4); 15 wks	WT mice + HFD (Fat 42% Cal.)	↓ Hepatic steatosis ↓ Insulin resistance (insulin x glucose) ↑ adiponectin	Yes
(Tomas et al., 2011a)	GLP-1 28-36 amide*	SC injections osmotic pumps; 11 wks	WT mice + HFD (Fat 60% Cal.)	↓ Hepatic steatosis ↓ Insulin resistance (insulin:glucose ratio)	Yes - fat mass only
(Sharma et al., 2011)	Liraglutide	IP injections; 4 wks	WT mice + ALIOS/FCS	↓ Hepatic steatosis ↓ ALT ↓ Oxidative stress (CHOP)	No
(Samson et al., 2011)	Exendin-4	SC osmotic pumps; 4 wks	WT mice+ HFD (Fat 60% Cal.)	↓ Hepatic steatosis ↓ Insulin resistance (↓glucose, ⇒insulin)	No
(Shirakawa et al., 2011)	Sitagliptin (DPP-4 inhibitor)	Oral (diet); 20 wks	GCK+/- mice + FFA/sucrose (oleic/linoleic)	↓ Hepatic steatosis ↓ Adipose hypertrophy	No
(Ben-Shlomo et al., 2011)	-	3X basal level of native GLP-1 in DPP-4-/- rats	DPP-4 -/- rat + HFD (Fat 60% Cal.)	↓ Hepatic steatosis ↓ ALT ⇒Insulin resistance (clamp technique)	No
(Trevaskis et al., 2012)	Exendin-4 analogue (AC3174)	SC osmotic pumps; 4 wks	Ob/Ob mice + ALIOS/cholesterol or HFD	↓ Hepatic steatosis ↓ ALT, not AST ↓ Hepatic fibrosis (biopsy/collagen-I)	Not when pair-fed**
(Lee et al., 2012)	Exendin-4	IP injections; 10 weeks	WT mice + HFD (Fat 40% Cal.)	↓ Hepatic steatosis ↓ NEFA ↓ Hepatic ballooning, inflammation	Yes
(Mells et al., 2012)	Liraglutide	IP injections; 4 weeks	WT + ALIOS/FCS	↓ Hepatic steatosis ↓ ALT (p=0.09) ↓ Insulin resistance (clamp) ↓ Blood pressure	No, but reduces adipose mass

**Table 1-8. The effect of GLP-1 based therapies in mouse models of NAFLD.**

Key: ALIOS, American Lifestyle-Induced Obesity Syndrome; Cal., Calories; CHOP, C/EBP homologous protein (key component of oxidative stress); FCS, fructose corn syrup; GCK+/- mice =  $\beta$ -cell-specific glucokinase haplo-insufficient diabetic mice; HDAd-Ex4, helper-dependent adenoviral (HDAd) vector for long-term expression of Ex4; HFD, high fat diet; IP, intraperitoneal; SC, subcutaneous; WT, wild-type. \*GLP-1(28-36) = a cleavage product of GLP-1(7-36), formed by neutral endopeptidases. \*\* pair-feeding is used to ensure equal food intake between the treatment and control groups.

Samson et al replicated the anti-steatotic effects of GLP-1 using a non-genetically modified, diet-induced obesity model. They fed wild-type (C57BL/J6) mice high fat diets and established chronic circulating exendin-4 levels by either genetic therapy or a continuous osmotic pump (Samson et al., 2008; Samson et al., 2011). HFD alone, however, has been criticised for not providing the full spectrum of NAFLD, most notably the absence of severe necroinflammation and hepatocyte ballooning. By mimicking an unhealthy fast-food diet, Tetri and colleagues devised the aptly named 'American Lifestyle-Induced Obesity Syndrome' (ALIOS) diet, on which wild-type mice develop severe necroinflammation, fibrotic changes and hepatic insulin resistance in the liver by 16-weeks (Tetri et al., 2008; Mells et al., 2012). The diet consists of a combination of high trans-fat (45% of calories derived from fat, 30% trans-fat) and water containing high fructose corn syrup. Using this model, two studies from Frank Anania's laboratory in the US have shown that liraglutide reduces hepatic steatosis, liver enzymes and endoplasmic reticulum oxidative stress after 4-weeks of intraperitoneal injections (Sharma et al., 2011; Mells et al., 2012).

#### 1.2.6.1.2 PROPOSED MECHANISMS OF GLP-1 IN HEPATIC STEATOSIS

Some rodent data support the possibility that the anti-steatotic action of GLP-1 may, to a certain extent, be independent of weight loss. In support of this notion, are the experiments that have used DPP-4 inhibition (Shirakawa et al., 2011) or deficiency (Ben-Shlomo et al., 2011) to moderately increase circulating levels of endogenous native GLP-1 and by doing so, highlight improvements in hepatic lipid accumulation in the absence of weight loss. Furthermore, by purposely incorporating pair-feeding to neutralise the effects of weight loss

Trevaskis and colleagues found significant reductions in hepatic steatosis in the mice treated with an analogue of exendin-4 (AC3174) compared to the saline-controls (Trevaskis et al., 2012).

In recent years, *in-vitro* studies that have utilised murine and human hepatocytes (either hepatocellular cancer cell lines and/or primary human cells), have added weight to the notion that GLP-1 might have a direct effect on hepatic lipid handling (Gupta et al., 2010; Ben-Shlomo et al., 2011; Sharma et al., 2011; Lee et al., 2012). Prior to treatment with GLP-1 in these experiments, hepatocytes were either fat-loaded in the presence of NEFA (mainly palmitic acid  $\pm$  oleic acid) in culture media or isolated from murine models of NAFLD (*discussed above*). Treatment with exendin-4 was seen to decrease intracellular lipid accumulation using both lipid quantification assays and oil-red O staining (Gupta et al., 2010; Sharma et al., 2011; Lee et al., 2012). Furthermore, studies have assessed the effect of exendin-4 treatment on the hepatic expression of genes (and protein in some studies) associated with fatty acid uptake,  $\beta$ -oxidation, hepatic DNL, and the export of very low density lipoprotein (VLDL); all of which are important to hepatocyte lipid handling. Using primary rat (Ding et al., 2006; Ben-Shlomo et al., 2011) *in vitro* studies have revealed that GLP-1/exendin-4 significantly reduces hepatic gene expression levels of ACC1 and FAS, which are rate-limiting enzymes involved in DNL. In addition, the gene expression sterol SREBP-1c that regulates the transcription of these key DNL enzymes is decreased with exendin-4 treatment (Ding et al., 2006; Ben-Shlomo et al., 2011; Gu et al., 2011). Reductions in gene expression reached 30-50% (FAS, SBREP-1c) on quantitative polymerase chain reaction (PCR) with 100nM GLP-1, whilst western blots confirmed FAS protein expression was reduced by

50% (Ben-Shlomo et al., 2011). In contrast, exendin-4 exerts the opposite effect on the expression of genes that are important for fatty acid breakdown. In either GLP-1 or exendin-4 treated hepatocytes four different laboratories have reported significant reductions in the gene expression of acyl-coenzyme A oxidase 1 (ACOX1) and carnitine palmitoyl transferase 1 (CPT1), which are key enzymes in peroxisomal and mitochondrial  $\beta$ -oxidation, respectively (Ding et al., 2006; Ben-Shlomo et al., 2011; Gu et al., 2011; Svegliati-Baroni et al., 2011). In support, the same studies reported reductions in the expression of peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ), which is a key regulator in the fore mentioned enzymes involved in fatty acid oxidation (Ding et al., 2006; Ben-Shlomo et al., 2011; Svegliati-Baroni et al., 2011).

These observations have been confirmed in-vivo with obesity murine models, from which liver tissue extracts were examined after a chronic period of GLP-1 elevation (via exogenous administration or genetic manipulation) (Ding et al., 2006; Lee et al., 2007; Samson et al., 2008; Ben-Shlomo et al., 2011; Shirakawa et al., 2011; Svegliati-Baroni et al., 2011). Mells and colleagues highlighted that liraglutide elicited the same changes with DNL and  $\beta$ -oxidation in mice fed the ALIOS diet (Mells et al., 2012). In addition, they found that long-acting GLP-1 analogues increased the gene expression of microsomal triglyceride transfer protein (MTTP), which facilitates lipid packaging and VLDL secretion in hepatocytes (Mells et al., 2012). Interesting, MTTP levels are suppressed in NASH models and by increasing MTTP, liraglutide might be averting the potential of lipid excess in the liver (Shindo et al., 2010; Mells et al., 2012). Most recently, GLP-1 agonists haven been shown to rescue hepatocytes from lipotoxicity in-vitro and in-vivo by promoting autophagy and subsequently protecting

against endoplasmic reticulum stress-induced cell death (Sharma et al., 2011). The latter, of which is thought to be a major player in the progression of hepatic steatosis to NASH fibrosis.

Although these studies offer interesting insights into the changes that occur in hepatocyte fatty acid transport and metabolism in response to GLP-1 therapy, there is a pressing need to confirm these genetic (and in part protein) findings using functional in-situ experiments (i.e. using stable isotope labeling).

#### 1.2.6.1.3 THE EFFECT OF GLP-1 ON INSULIN SENSITIVITY

The anti-hyperglycaemic action of GLP-1R agonists is thought to be largely due to the stimulation of insulin secretion and, to a lesser extent, by suppression of glucagon production. However, although the expression of GLP-1R in insulin-sensitive tissues, such as the liver (*discussed below*), adipose (Vendrell et al., 2011; Challa et al., 2012) and muscle (Delgado et al., 1995; Yang et al., 1998) remains conflicted, several animal studies (including depancreatized canines) have suggested that sustained treatment with GLP-1R agonists, in addition to its effects on insulin and glucagon secretion, is associated with improvements in insulin sensitivity (Sandhu et al., 1999; Young et al., 1999; Idris et al., 2002; Gedulin et al., 2005; Mannucci and Rotella, 2008; Arakawa et al., 2009). However, it is difficult to differentiate the indirect from the direct effects of GLP-1 on insulin sensitivity, because of concurrent changes in energy intake, weight, and other metabolic parameters; all of which can affect insulin sensitivity. Interestingly, when compared with pair-fed diabetic fatty

Zucker rats with matching HbA1C, fasting glucose, insulin, and lipids, Gedulin et al found that exendin-4 still increased insulin sensitivity (measured with euglycaemic clamps) by over 60% (Gedulin et al., 2005).

In the presence of hepatic steatosis, several groups have shown that treatment with GLP-1 reduces whole-body IR (Ding et al., 2006; Samson et al., 2008; Samson et al., 2011; Tomas et al., 2011a), as shown by improvements in basal levels of glucose in the absence of increases in insulin (i.e. HOMA-IR). Using the robust hyperinsulinaemic euglycaemic clamp technique, Mells and colleagues have recently shown that liraglutide improves insulin sensitivity at the level of the liver in mice fed the ALIOS diet (Mells et al., 2012). However, due to significant reductions in adiposity in this study, it is impossible to delineate whether this was due to direct actions of the GLP-1 analogue. However, Samson and colleagues have reported improved glucose clearance with exendin-4 treatment whilst on HFD, in the absence of changes in weight and circulating insulin; suggestive of weight-independent improvements in insulin sensitivity (Samson et al., 2011). Most studies, however, failed to incorporate isotope tracers to provide accurate measurement of glucose flux in the steady-state.

A detailed study by Lee and colleagues in 2007 utilised leptin-deficient (*Ob/Ob*) mice and a recombinant adenovirus expressing GLP-1 (rAd-GLP-1) to investigate tissue-specific effects of GLP-1 on insulin sensitivity (Lee et al., 2007). They performed hyperinsulinaemic euglycaemic clamps in the presence of isotope tracers of glucose (<sup>3</sup>H-glucose). In doing so, they reported that chronically elevated GLP-1 levels (4 weeks) significantly decreased basal endogenous glucose production compared to non-GLP-1 vector controls (i.e. improved



hepatic insulin sensitivity) (Lee et al., 2007). This was associated with reduced hepatic expression of glucose 6-phosphatase (involved in gluconeogenesis and glycogenolysis) and phosphoenolpyruvate carboxykinase (involved in gluconeogenesis). They elicited that this response was likely independent of GLP-1's reducing effect on glucagon, as there were no difference between treated and untreated mice in fasting glucagon levels (Lee et al., 2007); a finding consistent with previous human studies (Zander et al., 2002; Meneilly et al., 2003). However, they could not rule out the impact of weight loss on their findings, as it was highly significant in the GLP-1 treated insulin-resistant mice. The effect of GLP-1 on insulin signalling was supported by increased insulin-stimulated tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) and downstream protein kinase C (PKC) activity in both the liver and muscle of treated mice, with the later suggesting muscle insulin sensitization (Lee et al., 2007). It should be mentioned, that *in-vitro* studies in rat and human myocytes have failed to agree on the role of GLP-1 on glucose uptake and metabolism (Fürnsinn et al., 1995; González et al., 2005). Park et al later validated these findings over a longer duration (8 weeks) using exendin-4 in non-genetically manipulated rats fed a HFD (Park et al., 2010).

In contrast to Lee and others (Lee et al., 2007; Samson et al., 2011), Ben-Shlomo et al found that rats with elevated endogenous GLP-1 levels did not have higher levels of hepatic and peripheral sensitivity compared to weight matched controls (Ben-Shlomo et al., 2011). Even though they used the established euglycaemic clamp technique with isotopes glucose tracers in keeping with the fore mentioned, there are several possibilities for these study differences. First, they used normoglycaemic rodents, whereas Lee and others have studied those with insulin resistance. Second, the DPP-4  $-/-$  rats have elevated GLP-1 levels, but not

to the supra-physiological levels that are engineered via genetic therapy or exogenous administration. Third, they weight matched their rodents, whereas those in other studies have had considerable weight loss compared to the control-arms (Lee et al., 2007; Samson et al., 2011). Furthermore, even though the majority of in-vitro studies have reported that GLP-1/exendin-4 enhance phosphorylation Akt and activate key protein kinases in the insulin signaling pathway of hepatocytes (Redondo et al., 2003; Aviv et al., 2009; Gupta et al., 2010; Park et al., 2010; Svegliati-Baroni et al., 2011), this is not a uniform finding (Ben-Shlomo et al., 2011). Therefore, it remains unclear whether the anti-lipogenic effects of GLP-1 are dependent or independent of changes in insulin sensitivity.

In light of the growing importance of adipose dysfunction in NAFLD, understanding the potential effect of GLP-1 in adipose tissue is paramount. To date, however, the actions of such have been poorly studied *in-vitro* (Cignarelli et al., 2013). Studies performed in isolated rat and human adipocytes have demonstrated that GLP-1 may induce lipogenesis at picomolar concentrations or lipolysis when used at nanomolar doses (Ruiz-Grande et al., 1992; Perea et al., 1997; Villanueva-Peñacarrillo et al., 2001; Sancho et al., 2005; Vendrell et al., 2011). With regards to insulin sensitivity, GLP-1 has been shown to significantly increase insulin signaling in adipocytes by upregulation of phosphorylated IRS-1 and Akt (i.e. insulin signaling pathway), and in turn enhance glucose uptake (Egan et al., 1994; Wang et al., 1997; Gao et al., 2007; Vendrell et al., 2011). Some studies have highlighted differences between types of GLP-1, whereby exendin-4 and not GLP-1 increased insulin sensitivity in adipocytes lines (3T3) (Idris et al., 2002). Studies investigating the effect of GLP-1 on adipose tissue *in-vivo* are lacking in the literature.

#### 1.2.6.1.4 DOES GLP-1 MEDIATE ITS ACTION IN THE LIVER VIA ITS RECEPTOR (GLP-1R)?

Whether or not the GLP-1R is present in the liver and more specifically isolated hepatocytes remains controversial, with huge inconsistencies between research groups from around the world (Table 1-9). Most studies have investigated the presence of GLP-1R in hepatocyte/liver isolated from mice or rats, using a variety of techniques ranging from PCR, southern/western/northern blotting and in one case fluorescent in-situ hybridisation). The majority of these studies failed to identify the GLP-1R (Blackmore et al., 1991; Valverde et al., 1994; Wei and Mojsov, 1995; Bullock et al., 1996; Dunphy et al., 1998; Flock et al., 2007; Aviv et al., 2009; Liu et al., 2010; Tomas et al., 2011a).

To date, there have been only four studies that have used primary human tissue (Wei and Mojsov, 1995; Aviv et al., 2009; Gupta et al., 2010; Svegliati-Baroni et al., 2011), of which only two studies have used isolated human hepatocytes (Aviv et al., 2009; Gupta et al., 2010). Both of these studies were reported during the current research project (described in full in chapter 7.0). Gupta et al reported the presence of the GLP-1R in primary human hepatocytes using a wide range of techniques (Table 1-9) and highlighted that the GLP-1R is internalised on activation (Gupta et al., 2010). However, they provide very little data on the culture duration and origin of the primary hepatocytes used, which may alter gene expression. In contrast, Aviv and colleagues failed to identify the receptor in hepatocytes isolated from children and adults over 40 years (Aviv et al., 2009). Whether the findings of the later were due to a failure to insulin-starve their cells prior to RNA extraction requires further study.

Author/Year	Type of cell	Technique used	GLP-1R detected (Yes/NO)
<b>Rodent extracts</b>			
(Egan et al., 1994)	Rat whole liver	RT-PCR	Yes
(Campos et al., 1994)	Mouse whole liver	Northern blotting (mRNA)	Yes
(Ghiglione et al., 1985)	Primary rat hepatocytes	cAMP production	No
(Blackmore et al., 1991)	Primary rat hepatocytes	cAMP production	No
(Valverde et al., 1994)	Primary rat hepatocytes	cAMP production	No
(Bullock et al., 1996)	Rat whole liver	In-situ hybridization RT-PCR Nuclease protection assay	No
(Dunphy et al., 1998)	Rat whole liver	RT-PCR Southern blotting (DNA) Nuclease protection assay	No
(Ding et al., 2006)	Primary rat hepatocytes	cAMP production Western blotting (protein)	Yes
(Flock et al., 2007)	Primary mouse hepatocytes	cAMP production RT-PCR	No
(Tomas et al., 2011a)	Primary mouse hepatocytes	RT-PCR Western blotting (protein)	No
(Svegliati-Baroni et al., 2011)	Rat whole liver; Primary rat hepatocytes	RT-PCR Western blotting (protein)	Yes
(Ben-Shlomo et al., 2011)	Primary rat hepatocytes (from DPP-4 -/-)	cAMP (no experiments for GLP-1R per se)	Yes
<b>Human extracts</b>			
(Wei and Mojsov, 1995)	Whole human liver	Nuclease protection assay	No
(Aviv et al., 2009)	Primary human hepatocytes	Western blotting (protein) RT-PCR cAMP production*	No*
(Gupta et al., 2010)	Primary human hepatocytes** Hepatoma-cell lines (Huh7; HepG2)	RT-PCR Western blotting (protein) IF/Confocal microscopy Bioluminescence assay	Yes
(Svegliati-Baroni et al., 2011)	Human whole liver (biopsies from patients)	RT-PCR Western blotting (protein)	Yes
(Lee et al., 2012)	Hepatoma-cell lines (Huh7; HepG2)	RT-PCR Western blotting (protein)	Yes

**Table 1-9. Data from human and rodent studies for the absence or presence of G-protein coupled GLP-1R (+/- cAMP) in the whole liver tissue and/or hepatocytes.**

\*Aviv et al did not identify the gene or protein expression of GLP-1R (data not shown in manuscript), but found a G-protein coupled receptor response ( $\uparrow$ cAMP). \*\*no information provided on the donor status of the hepatocytes. Key: cAMP, cyclic adenosine monophosphate; DNA, deoxyribonucleic acid; DPP-4, dipeptidyl peptidase-4; IF, immunofluorescence; mRNA, messenger ribonucleic acid; RT-PCR, real-time polymerase chain reaction.

### 1.2.6.2 Clinical studies and reports

To date, human studies investigating the effect of GLP-1R agonists on the human liver have been limited to individual case reports (Tushuizen et al., 2006; Ellrichmann et al., 2009), an uncontrolled open-label retrospective study in patients with type 2 diabetes (Buse et al., 2007) and a solitary case series (Kenny et al., 2010).

Tushuizen *et al* reported the case of a 59 year old with type 2 diabetes found to have fatty liver disease (alcohol consumption not reported) with MRI spectroscopy (Tushuizen et al., 2006). Percentage hepatic steatosis decreased from 16% to 4% after 44 weeks therapy with 10mg BD exenatide in combination with long-standing metformin. This was accompanied by reductions in liver enzymes, namely ALT (46 to 20 IU/L) and AST (18 to 13 IU/L), and concomitant systemic metabolic improvements (4.7% body weight loss, triglycerides -0.77 mmol/L). Three years later, Ellrichmann and colleagues described the case of a 49 year old obese patient with type 2 diabetes and co-existing biopsy proven HCV (genotype 1a) and NASH (NAS 6/8; Kleiner fibrosis stage 3) (Ellrichmann et al., 2009). The addition of 10mg BD exenatide to his diabetic treatment regimen of 1g BD metformin, after 2 previous failed attempts to successfully treat the HCV with standard Peg-interferon and ribavarin, resulted in a 40% reduction in hepatic steatosis on repeat biopsy. There was no mention of changes to the other features of NASH, but the patient had significant reductions in weight, HbA1c and liver enzymes. The reduction in hepatic steatosis and insulin resistance may provide an explanation as to why sustained elimination of HCV was witnessed during 44 weeks follow-up after a third course of anti-viral therapy (Ellrichmann et al., 2009).

In support of these findings, Buse and colleagues retrospectively analysed data from a large open-labelled study (n=974) of obese subjects with type 2 diabetes (Buse et al., 2007). Patients had been on long standing oral therapy with either metformin and/or sulphonylurea prior to being commenced on exenatide for two years. Patients with elevated ALT at baseline (151/283 [53%]) had a significant reduction of ALT (-11 IU/L) from baseline 38 (IU/L), of which 39% achieved normal ALT levels (as defined by the Prati cut-off (Prati et al., 2002); Female  $\leq 19$  IU/L; male  $\leq 30$  IU/L) by 2 years. In keeping with the case reports, these changes were accompanied by reductions in weight (-4.7 kg) and HbA1c (-1.1%). A further report from the same study group highlighted that these metabolic changes were sustained by extending the therapy out to 3 years in total (Klonoff et al., 2008). This study was limited, however, by the lack of a placebo comparison and the fact that NAFLD was not well defined at baseline. Furthermore, the study was powered on glycaemic control and not markers of liver injury.

In 2010, a prospective case series of eight biopsy-proven NAFLD patients with type 2 diabetes provided encouraging support to the beneficial hepatic effects of exenatide BD previously seen in rodent models and retrospective studies (Kenny et al., 2010). Three out of the eight patients had histological improvements (including reductions in hepatocyte ballooning) after 28 weeks of treatment. Significant reductions were also reported in weight (-4.9 kg), fasting glucose (-1.5 mmol/l), HbA1c (-1.0%) and ALT (-24 U/l). As acknowledged by the authors themselves, the results should be interpreted with some caution in light of the small sample size, lack of control-arm and short follow-up phase (Kenny et al., 2010). At present, however, there are a lack of powered, randomised, placebo-controlled trials in the

literature that have investigated the effects of a GLP-1R agonist in patients with biopsy confirmed NASH.

### **1.3 Summary**

NAFLD and its increased risk of CVD have emerged as a growing public health problem worldwide. Despite the fact that our knowledge of the pathogenesis, diagnosis and prognosis of NAFLD has expanded over the last decade, there is still no universally accepted safe, pharmacological therapy for NASH. Collectively, the pre-clinical and clinical data presented above suggest that GLP-1 based therapy might have direct and indirect therapeutic benefits in patients with NASH and their associated CVD risk. The fact that they have the ability to improve glycaemic control (and potential insulin resistance) and induce weight loss, in addition to possible direct anti-steatotic actions in the liver makes them an exciting therapeutic option for study in NASH, which warrants further investigation.

The main hypothesis of this project was that GLP-1 analogues are safe and have direct and indirect therapeutic benefits in patients with NASH.

## 1.4 Aims of the project

The main aim of this project was to understand the therapeutic role of GLP-1 based therapies in NASH. In addition to answering this it was deemed important to investigate the clinical burden and severity of NAFLD in primary care; knowledge of which was lacking in the UK. Furthermore, in order to understand the potential mechanisms of GLP-1 in NASH, it was first necessary to elicit the sites and extent of insulin resistance in patients with the condition. Therefore, the specific objectives of the project were:

- ❖ To determine the presence and disease severity of NAFLD in a large, prospective primary care population [**Chapter 2**].
- ❖ To meta-analyse the safety and efficacy of the long-acting GLP-1 analogue, liraglutide, on liver parameters in a large clinical trial population of patients with type 2 diabetes [**Chapter 3**].
- ❖ To design and perform a phase II, multi-centre, double-blinded, placebo-controlled randomised clinical trial (LEAN) in order to investigate the safety and efficacy of liraglutide in patients with biopsy-proven NASH [**Chapter 4**].
- ❖ To determine the relative contribution of tissue-specific insulin sensitivity (hepatic, muscle, adipose) and hepatic DNL in patients with biopsy-proven NASH [**Chapter 5**].
- ❖ To determine the effect of 12-weeks treatment of 1.8mg liraglutide (vs. placebo controls) on tissue-specific insulin resistance, hepatic DNL and dysfunctional adipose tissue in patients with NASH [**Chapter 6**].
- ❖ To determine the direct anti-steatotic effects of GLP-1 analogues in the liver (*in-vitro*) [**Chapter 7**].



## **CHAPTER 2: PRESENCE AND SEVERITY OF NONALCOHOLIC FATTY LIVER DISEASE IN A LARGE PROSPECTIVE PRIMARY CARE COHORT**

### **2.1 Introduction**

The incidence of liver disease is rising throughout the world and now accounts for 1.5% of deaths in the UK ([www.statistics.gov.uk](http://www.statistics.gov.uk)). In parallel with this there has been a year on year rise in the number of LFT profiles carried out in UK primary care practices (from 62300 to 109619/year between 2002 and 2010; University Hospital Birmingham (UHB) laboratories audit, UK). Primary care practitioners (PCPs) are thus commonly faced with the scenario of abnormal LFTs in patients in whom there are no clinical risks, signs or symptoms of liver disease. NAFLD is now recognized as the most common cause of hepatic dysfunction in general population (Clark et al., 2003; Bedogni et al., 2005), however this is yet to be confirmed in primary care practice. Furthermore, because of the indolent asymptomatic nature of NAFLD, identifying those with advanced disease in whom specific interventions may be required remains a clinical challenge in primary care.

The prevalence of NAFLD has risen markedly to 14 to 34% of the general-population in Europe (Bedogni et al., 2005; Caballería et al., 2007), Asia (Hamaguchi et al., 2005), and America (Browning et al., 2004) in recent years. Whilst patients with simple NAFLD are believed to have benign disease, there is now clear evidence that those who have progressed to NASH and fibrosis are at a much higher risk of developing HCC, liver failure and death (Bugianesi et al., 2002; Ekstedt et al., 2006). The majority of data describing the

severity of liver fibrosis in NAFLD arises from selected populations in secondary referral centres (Hultcrantz et al., 1986; Daniel et al., 1999; Skelly et al., 2001; Ekstedt et al., 2006; Angulo et al., 2007; Neuschwander-Tetri et al., 2010; Söderberg et al., 2010). In a large UK prospective study, Skelly et al demonstrated that 18% (23/120) of biopsy confirmed NASH patients had significant fibrosis after presenting to their secondary care centre with unexplained abnormal LFTs (Skelly et al., 2001). This and other such studies (Hultcrantz et al., 1986; Daniel et al., 1999) included patients in whom the decision to refer had been made on clinical grounds by PCPs/consultant colleagues and were then rigorously screened in liver clinics for other disease aetiologies prior to proceeding to liver biopsy. These studies are therefore influenced by ascertainment bias and may overestimate the severity of NAFLD emerging from primary care.

It is currently expected with the alarming growth of obesity and type 2 diabetes that the burden of NAFLD on primary care and liver services will continue to rise in the UK (Ahmed et al., 2010). To date, no studies have determined the underlying disease severity of NAFLD in primary care. PCPs remain at the forefront of identifying the patients with advanced NAFLD who require further evaluation, closer surveillance for complications (and interventions where appropriate) and stricter lifestyle modifications. By investigating a large UK primary care sample of patients with incidental abnormal LFTs and absent clinical features of liver disease, this study is the first of its kind to determine the presence and disease severity of silent NAFLD in a primary care setting.

## **2.2 Methods**

### **2.2.1 Study population**

Birmingham and Lambeth Liver Evaluation Testing Strategies (BALLETS) is a prospective study of patients with an incidental finding of abnormal LFTs in primary care funded by NIHR Health Technology Assessment programme (Lilford et al., 2013a). Patients were prospectively recruited from 11 primary care practices from Birmingham and Lambeth areas, between 2006 and 2008. The primary aim of the BALLETS study was to assess the clinical utility of abnormal LFTs in patients in whom liver disease was not suspected clinically by the PCP (Lilford et al., 2013b). St. Thomas' Hospital Research Ethics Committee approved the study and all study participants gave signed informed consent to be included.

This current cross-sectional sub-study utilizes baseline data from patients enrolled in the BALLETS study from the eight primary care practices within the Birmingham region only. PCPs from participating practices reviewed all new incidental abnormal LFT results arising from their practices in patients in whom the clinical suspicion of underlying liver disease was absent or low. Patients over eighteen years old were eligible for the sub-study if one or more LFT analyte was abnormal and there was no previous documented history of liver disease, intravenous drug use and/or alcohol-related health problems. Current signs or symptoms suggestive of liver disease, pregnancy and a diagnosis of disseminated malignancy were also considered exclusion criteria. Eligible patients who consented for the study completed an interview during which current illnesses, past medical history, alcohol consumption, socio-

demographic details, and drug history were recorded. Reasons for the original LFTs being ordered by the PCP were also recorded. Patient's height, weight and waist circumference were measured. All patients had a repeat set of LFTs and a full serological liver aetiology screen (viral, genetic and autoimmune) at the study visit. An abdominal USS was obtained in the fasted state using an ultrasound machine (TITAN® Sonosite) operated by one of five (10 to 30 years experience) abdominal sonographers. All scans were recorded on tape and 50 of these were selected at random and validated by a consultant radiologist (Olliff S).

PCPs were sent a consolidated report of all study investigations. The study team recommended to the PCP the need for a hepatology referral to the tertiary liver clinic (UHB) in the event of one of the following: 1) positive serological liver aetiology screen; 2) sonographic features of cirrhosis (coarse echotexture, irregular contour), space occupying liver lesion(s) or biliary duct dilatation. All liver clinic letters were retrospectively reviewed (until 1<sup>st</sup> May 2010) to identify which of these diagnoses were followed up and confirmed by a liver specialist (Table 2-1).

Liver Disease Aetiology	Hepatology referral criteria	Referral criteria met n	Liver specialist follow-up n (%)	Diagnosis Confirmed n (%)	Diagnosis Information
Hepatitis B virus (HBV)	+ve HBV surface antigen	8	8 (100%)	8 (100%)	Positive HBV surface antigen
Hepatitis C virus (HCV)	+ve HCV antibody	2	2 (100%)	2 (100%)	Detectable HCV RNA. Genotype 3 (n=1) Genotype indeterminate (n=1)
Autoimmune Hepatitis (AIH)	+ve anti-smooth muscle antibody (> 1:40) and AST or ALT > 100 U/L	0	-	-	Liver biopsy
Primary Biliary Cirrhosis (PBC)	Positive anti-mitochondrial antibody (> 1:40)	12	12 (100%)	9 (75%)	M2-subtype positive and predominant serum ALP and GGT abnormality. Diagnosis excluded (n=3).
Primary Sclerosing Cholangitis (PSC)	Abnormality of bile ducts on ultrasound.	3	2 (67%)	2 (67%)	Liver biopsy (n=1). Magnetic Resonance Cholangio-pancreatography (n=1). Did not attend investigation (n=1).
Genetic Haemo-chromatosis	Transferrin saturation > 50%	34	28 (82.4%)	10 (29.4%)	Genetic phenotyping revealed: C282Y homozygotes (n=5), H63D homozygotes (n=1), Compound heterozygotes. (n=4), Carrier status (n=2), Normal (16).
Alpha 1 Antitrypsin Deficiency (A1AD)	Serum alpha 1 antitrypsin level < 1.5g/L and ZZ/ZS/SS phenotypes detected by isoelectric focusing	2	2 (100%)	2 (100%)	ZS phenotype (n=1) SS phenotypes (n=1).
Wilson's disease	Serum caeruloplasmin < 0.2 g/L	20	3 (4.4%)	0 (0%)	24hr urine copper levels and liver biopsy. Excluded (n=3)
Liver malignancy	Suspicious space-occupying lesion(s) on ultrasound	3	3 (100%)	1 (33.3%)	Liver Metastases (n=1). Amoebic liver abscess (n=1). Incidental NASH cirrhosis on biopsy, no malignancy (n=1).
Alcohol or non-alcoholic induced cirrhosis	Cirrhotic features (coarse echotexture, irregular outline) on ultrasound	2	2 (100%)	2 (100%)	Alcohol induced cirrhosis (n=2)

**Table 2-1. Hepatology referral criteria, follow up and liver disease diagnostic rates.**

Incomplete aetiology screen and follow-up was classified as 'unexplained' for study documents. Alcohol excess and non-alcoholic fatty liver on ultrasound were not included in the hepatology referral criteria at the time of study design.

### 2.2.2 Data Definitions

The sub-study LFT profile consisted of ALT, AST, GGT, alkaline phosphatase (ALP), total bilirubin, and albumin measurements. Seven of the eight Birmingham practices sent samples to a central laboratory at UHB, whilst the remaining practice sent samples to the laboratory of Russell's Hall Hospital. Initial LFTs requested by the PCP were used as a criterion for study entry, whereas the repeat LFTs undertaken at the study visit were performed to increase the likelihood of a complete panel of the 6 analytes listed and to avoid analyte selection bias that may have occurred in the primary care practice. The analytes were classified as normal or abnormal based on reference ranges specific to each of the two individual laboratories, which are compliant with International Quality Control Standards. The full blood liver aetiology screen consisted of HBV surface antigen, HCV antibody, caeruloplasmin, iron and transferrin saturation, A1AT, anti-smooth muscle and anti-mitochondrial antibodies.

BMI was defined as weight in kilograms divided by the square of the height in meters ( $\text{kg/m}^2$ ). Obesity was defined as  $\text{BMI} \geq 30 \text{ Kg/m}^2$ . Alcohol intake was reported as standard units (1 unit = 10g Alcohol) of alcohol consumed on average per week in the 6 months prior to recruitment. Mild (female 1-7 units, male 1-11 units/week) and moderate (female 8-14 units, male 12-21 units/week) alcohol consumption were defined as drinking within the current UK health guidelines (female  $\leq 14$ , male  $\leq 21$  units/week; British Medical Association 1995). At-risk alcohol consumption was defined as exceeding these guidelines.

For the purposes of this sub-study, type 2 diabetes was defined in patients with a documented history of the disease or a recorded drug history of anti-diabetic medication. Hypertension was defined as a past medical history of the disease or a current recorded drug history of two or more anti-hypertensive medications.

The diagnosis of NAFLD was based on the following criteria: (1) sonographic diagnosis of fatty liver, defined as diffusely increased liver echogenicity (> right renal parenchyma) with vascular blurring; (2) a negative history of alcohol consumption exceeding current UK health guidelines; and (3) exclusion of liver disease of other aetiology including drug-induced, autoimmune, viral hepatitis, cholestatic, metabolic and genetic liver disease.

### **2.2.3 NAFLD Fibrosis Score (NFS)**

NFS (Angulo et al., 2007) is a simple non-invasive scoring system designed to identify or exclude advanced fibrosis (classified as Kleiner stages F3 and F4 (Kleiner et al., 2005)) in patients with an established diagnosis of NAFLD on imaging. The NFS was developed and validated by Angulo *et al* (Angulo et al., 2007) in over 700 liver biopsy-proven patients with NAFLD and is routinely used in liver clinics to select those at risk of disease progression and HCC. The NFS utilises a number of simple clinical and laboratory independent predictors of advanced liver fibrosis. The equation is as follows (Angulo et al., 2007):

$$\text{NFS} = -1.675 + 0.037 \times \text{age (years)} + 0.094 \times \text{BMI (kg/m}^2\text{)} + 1.13 \times \text{IFG/diabetes (yes=1, no=0)} \\ + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{platelet count (x10}^9\text{/L)} - 0.66 \times \text{albumin (g/dL)}$$

The low cut-off score ( $< -1.455$ ) has a NPV of 88 to 93% and the high cut-off score ( $> +0.676$ ) has a PPV of 79 to 90% for the presence of advanced fibrosis in NAFLD in secondary care populations (Angulo et al., 2007; McPherson et al., 2010). The NFS was calculated retrospectively using the web-based calculator (<http://NAFLDscore.com>).

As the original BALLETS study protocol did not incorporate a platelet count, retrospective data collection of the electronic haematology laboratory archive at the UHB enabled platelet counts within 6 months of patient enrolment to be recorded. To avoid false positive or false negative NFS, the scoring system was not applied to participants with a past medical history of platelet disorders, on myelosuppressive medications or an active systemic-inflammatory disease.

#### **2.2.4 Statistical Analysis**

Descriptive statistics were applied to characterize the whole study cohort and the identified NAFLD group. Continuous clinical and laboratory variables are reported as medians and interquartile ranges (IQR) as all variables had a non-parametric distribution on D'Agostino and Pearson Omnibus Normality testing (GraphPad Prism 5). Categorical variables are reported as numbers and percentages. Due to a variation in normal reference ranges between the two laboratories utilized for the initial PCP LFT samples, blood results from Russell's Hall Hospital (n=89 patients) were standardised to the central laboratory reference ranges at UHB using the proportion of the upper (or lower with albumin) limit of normal.



## 2.3 Results

A total of 1118 primary care patients were included. The PCPs reason for the LFT requests are shown in Table 2-2. The majority (38%; 424/1118) of these resulted from routine chronic disease check-ups. In 4.5% (50/1118) of cases no reason was recorded. Liver aetiology screen and ultrasound were successfully completed in 98% (1101/1118) of patients at the study visit. There was a 100% agreement between the consultant radiologist and the study sonographers in reporting the presence or absence of fatty liver on USS in 50 randomly selected cases. Study demographics and characteristics are summarised in Table 2-3.

Documented reason	Percentage (n)
Diabetes review	18.0 (201)
Non-specific routine bloods	15.2 (171)
Hypertensive disease review	11.4 (128)
Gastrointestinal symptoms (excluding liver-specific)	10.0 (112)
Generalised fatigue or tiredness	6.2 (69)
Cardiovascular disease review	4.7 (53)
Medications review (non-specific)	4.5 (50)
Hyperlipidaemia disease review	3.8 (42)
Neurological symptoms (inc. confusion)	2.7 (31)
Musculoskeletal symptoms (i.e. joint pain)	2.4 (27)

**Table 2-2. The ten most commonly recorded reasons for why the LFTs were undertaken by the PCP.**

Values are percentages (numbers). Percentages include all values (n=1118). Other reasons accounted for 20.9% (234).

Characteristics	Total (n =1118)	NAFLD (n =295)
Median (IQR) age (years)	60 (48-70)	58 (49-66.7)
Gender		
Male	56 (628)	56.6 (167)
Female	44 (490)	43.4 (128)
Ethnicity (%)		
White	83.9 (938)	84.1 (248)
African-Caribbean	3.9 (44)	2.0 (6)
Asian/Arabic	8.1 (90)	9.8 (29)
Mixed/other	1.3 (15)	1.7 (5)
Unknown	2.8 (31)	2.4 (7)
<b>Alcohol consumption cut-offs</b>		
Abstinence	42.5 (475)	56.9 (168)
Mild	20.8 (232)	28.1 (83)
Moderate	10.5 (117)	14.9 (44)
At-risk	26.3 (294)	0 (0)
<b>Metabolic Phenotypes</b>		
Type 2 diabetes	23.5 (263)	38.6 (116)
Hypertensive Disease	43.2 (483)	45.4 (134)
Obesity	40.7 (455)	60.3 (179)
Median (IQR) measured BMI (Kg/m <sup>2</sup> )	28.7 (25.3-33.1)	31.5 (28.1–35.8)
Median (IQR) waist circumference (cm)		
Male	103 (95-112)	107 (101-115)
Female	96 (85-109)	107 (96-115)

**Table 2-3. Demographics and characteristics of study participants (*left*) and those identified with NAFLD (*right*).**

Values are percentages (numbers) unless stated otherwise. Percentages do not include missing values.

### 2.3.1 Causes of abnormal LFTs

The cause of abnormal LFTs was identified in 54.9% (614/1118) of cases (Table 2-4). Detailed testing for viral, genetic and autoimmune causes yielded 33 diagnoses (3.0%). NAFLD was identified as the commonest cause of abnormal LFTs accounting for 26.4% of all cases, exceeding alcohol excess (25.3%). The demographics and metabolic parameters of the identified NAFLD group are summarised in Table 2-3.

Cause	% (n)	GGT [U/L]	ALT [U/L]	AST [U/L]	ALP [U/L]	Bilirubin [μmol/L]	Albumin [g/L]
<b>NAFLD</b>	26.4 (295)	59 (41-88)	38 (27-54)	30 (23-40)	206 (167-266)	9 (6-12)	45 (43-47)
<b>At-risk alcohol intake</b>							
Non-Fatty liver	14.0 (156)	69 (46-115)	30 (22-44)	28 (22-35)	190 (159-238)	10 (7-13)	46 (44-48)
Fatty liver	11.3 (126)	81 (52-148)	46 (33-65)	36 (28-49)	178 (150-218)	9 (8-13)	47 (45-49)
<b>PBC</b>	0.81 (9)	99 (45-186)	15 (20-31)	27 (25-36)	396 (337-463)	7 (6-13)	43 (42-45)
<b>HBV</b>	0.72 (8)	53 (32-418)	92 (49-156)	62 (26-97)	184 (147-242)	8 (5-15)	46 (43-52)
<b>Haemochromatosis</b>							
Homozygote [C282Y or H63D]	0.54 (6)	73 (31-166)	59 (43-79)	39 (32-56)	202 (158-382)	8 (5-23)	46 (45-48)
Comp. heterozygote [C282Y+H63D]	0.36 (4)	56 (25-458)	51 (54-149)	25 (42-238)	121 (75-135)	12 (5-21)	51 (45-53)
<b>Other</b> (inc. cancer, drug, abscess)	0.36 (4)	85 (27-179)	29 (17-58)	31 (18-44)	273 (191-368)	12 (7-18)	44 (39-48)
<b>HCV*</b>	0.17 (2)	x (34, 452)	x (151, -)	x (101, 70)	x (514,214)	x (8,8)	x (48,47)
<b>PSC*</b>	0.17 (2)	x (-, 600)	x (51, 212)	x (33, 124)	x (176,990)	x (12,10)	x (47,46)
<b>A1AT*</b>	0.17 (2)	x (59, 62)	x (41, 50)	x (24, 25)	x (161,138)	x (11,12)	x (48,50)
<b>Unexplained group</b>	45.1 (504)	56 (33-91)	26 (19-38)	26 (22-33)	202 (162-274)	9 (6-13)	45 (43-47)

**Table 2-4. Causes of incident abnormal LFTs**

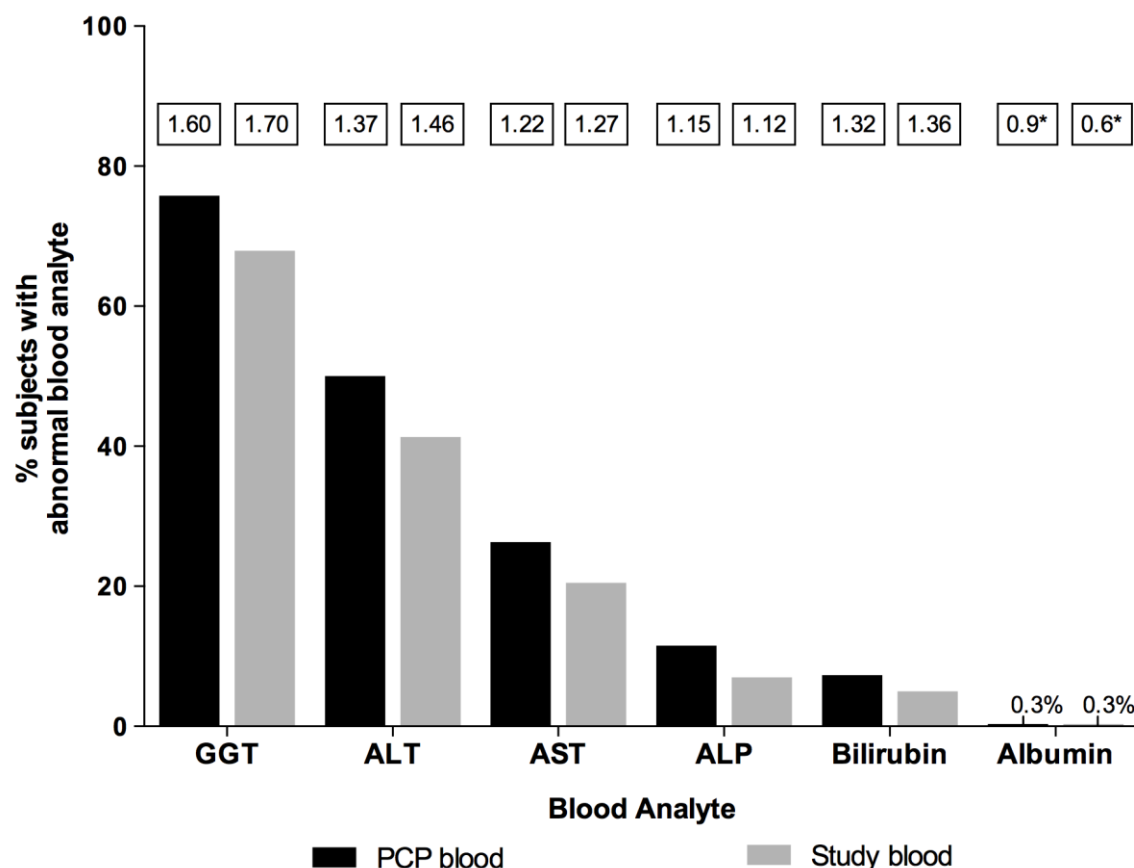
Percentages include all values (total n=1118). LFT analyte (inclusive of normal and abnormal values) from study visit are expressed as medians (IQR or \*single values).

There were no reported cases of cirrhotic appearances or ascites on USS in the NAFLD cohort. Splenomegaly ( $\geq 13$  cm) was reported in 7.8% (23/295) of NAFLD cases, albeit only marginally enlarged (median 13.6 cm, IQR 13.2-14.0). Two or more abnormal LFT analytes were present in 40.7% of NAFLD subjects (120/295), with the remainder having a single analyte abnormality (59.3%;175/295) on PCP sampling. GGT was the most common LFT abnormality in the NAFLD cohort (75.7%;197/260), with a median value 1.6 times the upper

limit of normal (Figure 2-1). Median time difference between bloods ordered by the PCP and the study visit was 30 days (IQR 18 – 51).

At-risk alcohol consumption was reported in 25.3% (282/1118). The majority of at-risk alcohol consumers were male (73.4%; 126/282) and drank a significant greater amount of alcohol (units per week) than females (median 42 (IQR 30-56) versus 29 (IQR 21-46), Mann-Whitney U test =  $p < 0.001$ ). An echo-bright fatty liver was identified with USS in 44.7% (126/282) of subjects who consumed at-risk levels of alcohol. The majority of excess drinkers (87%; 110/126) had a BMI greater than 25 kg/m<sup>2</sup>. USS identified cirrhotic appearances in two cases (one with splenomegaly; 15cm) of at-risk alcohol consumption. Tertiary liver specialists confirmed the diagnosis of compensated alcohol-induced cirrhosis.

No cause for LFT abnormality was identified in the remainder of study subjects (45.1%; 504/1118). Liver disease could not be ruled out in 8.1% (41/504) of unexplained cases due to incomplete liver aetiology screen (n=10), USS (n=7) and absence of referral to liver specialist/patient non-attendance after a positive liver aetiology screen test (bile duct dilatation, n=1; transferrin saturation >50%, n=6; low caeruloplasmin, n=17). LFTs normalised between PCP and study visit sampling (median 30 days, IQR 18-63) in 19.9% (92/463) of unexplained cases with a completed USS and liver aetiology screen. Metabolic risk factors in the unexplained abnormal LFT group included obesity (30.5%, 154/504), diabetes (19.0%, 96/504) and hypertension (41.3%, 208/504). Of note, 18.5% (95/504) had co-existing obesity with either diabetes and/or hypertensive disease.



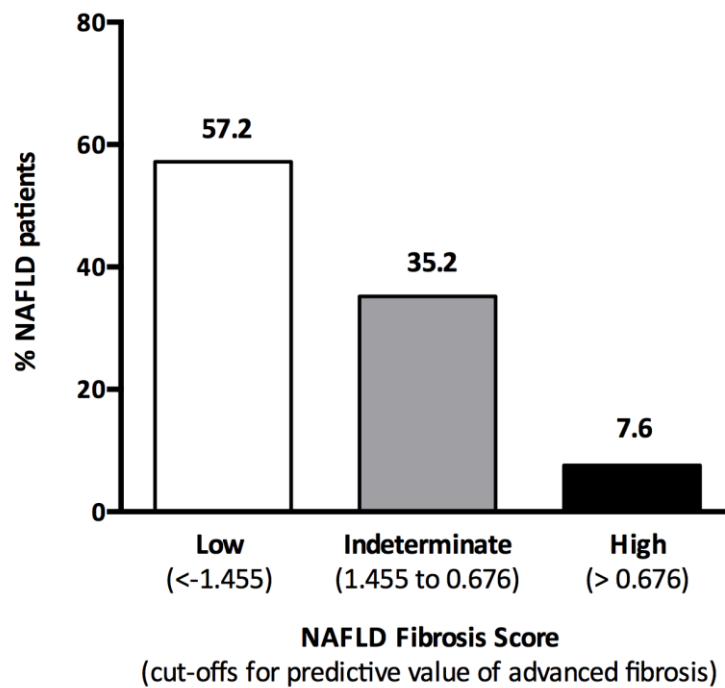
**Figure 2-1. Frequency and extent of LFT abnormalities in the identified NAFLD cohort.**

Percentages do not include missing values. The extent of the LFT abnormality is expressed as a proportion of the upper (or lower\*) limit of normal [median values reported in boxes].

### 2.3.2 Disease severity in the cohort of patients with NAFLD

To calculate the severity of NAFLD in this cohort we retrospectively applied the NFS (Figure 2-2). The score was calculated in 236 of the 295 patients who met the diagnostic criteria for NAFLD. The NFS was not calculated in the remaining 59 patients with NAFLD as a result of incomplete records of blood platelets (n=50), BMI (n=5) and AST/ALT ratio (n=4). A high NFS ( $> +0.676$ ) was found in 7.6% (18/236) of patients with NAFLD, suggesting the presence of underlying advanced liver fibrosis (Stages F3/F4 on Kleiner classification (Kleiner et al.,

2005)). Advanced fibrosis was predicted to be absent in the majority of NAFLD subjects with a low NFS ( $<-1.455$ ) being calculated in 57.2% (135/236). The presence of advanced fibrosis, however, could not be confidently excluded in 35.2% (83/236) of the NAFLD patients who scored an indeterminate value with the NFS ( $-1.455$  to  $+0.676$ ).



**Figure 2-2. NAFLD Fibrosis Scores in patients that met the diagnostic criteria for NAFLD.**  
Percentages do not include missing values

## 2.4 Discussion

This large primary care study highlights that definite NAFLD accounts for over 25% of incidental abnormal LFTs in primary care consultations, in which the consulting PCP's suspicion of underlying liver disease is low or absent. In contrast, only 3.0% of all study patients had a specific viral (HBV/HCV), genetic or autoimmune disease identified on thorough study testing. Application of a simple, non-invasive scoring system suggests that undetected advanced liver fibrosis is present in 7.6% and absent in 57.2% of the NAFLD patients. Incidental abnormal LFTs were most commonly encountered during routine chronic disease reviews (38% cases), including diabetes, hypertension and cardiovascular disease. This study is the first of its kind to report the severity of NAFLD in patients with incidental abnormal LFTs in primary care.

Our study evaluated a PCP-based population with abnormal LFTs (whom had no obvious cause/history of liver disease at the initial PCP consult) rather than a population volunteered from the general community. Nonetheless, the frequency of NAFLD (26%) identified in our study is within the wide range (14 to 31%) previously reported in general population studies carried out in Italy (Bedogni et al., 2005), Spain (Caballería et al., 2007), Asia (Hamaguchi et al., 2005) and America (Browning et al., 2004). The variation in reported frequencies may be influenced by ethnic diversity (Browning et al., 2004; Petersen et al., 2006) and differences in study methodologies. These include variable alcohol thresholds that define NAFLD, lack of consistency in screening for other disease aetiologies and variation in risk stratification for liver disease at study enrolment. All the studies nevertheless confirm the strong association

between NAFLD and components of the metabolic syndrome (Marchesini et al., 2001; Hamaguchi et al., 2005) the prevalence of which has increased rapidly worldwide (Ahmed et al., 2010). The high proportion of patients with diabetes (38.6%), obesity (60.3%) and hypertension (45.4%) in the NAFLD group in our study is in keeping with population-based studies (Bedogni et al., 2005).

The suspected proportion of advanced fibrosis within our NAFLD cohort is 7.6%. Additionally, from experiences in hospital care (Angulo et al., 2007; Wong et al., 2008; McPherson et al., 2010) we predict that a sub-set of the 35.2% of patients with an indeterminate NFS may also have advanced fibrosis. There are currently no data on the severity of NAFLD in UK primary care. The most relevant studies that best reflect low-risk populations are restricted to biopsy findings in living-related liver donors, in which the prevalence of NASH (+/- fibrosis) ranges from 1.1% in Japan (Yamamoto et al., 2007) to 18.5% in the US (Tran et al., 2006). The latter figure is likely to be an overestimate due to the lack of detail on alcohol consumption and full liver aetiology screening in liver donors. Secondary/tertiary centre studies of variable size (range 118 to 733) and Caucasian predominance have reported that 11% to 27% of patients with biopsy-proven NAFLD and elevated aminotransferases have advanced (stages 3/4) fibrosis (Ekstedt et al., 2006; Harrison et al., 2008; Wong et al., 2008; Angulo, 2010; Söderberg et al., 2010). The higher rates of advanced fibrosis reported in these liver specialist centres are likely to be due to referral/sampling bias.



The current study has several unique strengths. First, this is the largest prospective cohort of primary care patients with clinically unsuspected liver disease and incidental abnormal LFTs to be reported. Second, this is the first study to apply the non-invasive NFS to identify patients with advanced NAFLD fibrosis in primary care that are most in need of intensive lifestyle modifications and surveillance for liver-related complications (e.g. HCC detection). Third, the detailed assessment of the liver aetiology screen (alcohol/drug data, serology, genetics and USS imaging) undertaken and high completion rate (98%) has meant that a cause for abnormal LFT was identified in the majority of cases (55%). Previous large-scale population-based retrospective analyses of abnormal LFTs have been limited by the absence of USS (Clark et al., 2003) and the lack of information on alcohol and measured anthropometry (McLernon et al., 2009) to accurately describe the presence of NAFLD. The high rate of liver disease identification in our patient sample that PCPs perceived as a low risk group may also be explained by the fact that GGT, which has the highest reported sensitivity for liver disease above other LFTs (McLernon et al., 2009), was the commonest LFT abnormality. The finding of an elevated GGT in more than 70% of the NAFLD group as opposed to ALT (51.0%) and AST (26.2%) has not previously been reported in adult NAFLD patients. This finding has also been reported in paediatric NAFLD (Feldstein et al., 2009a).

One limitation of the NFS is that its application is only validated against liver biopsy in NAFLD patients attending hospital (Angulo et al., 2007; Wong et al., 2008; McPherson et al., 2010), and so it is possible that the severity of NAFLD may be over-estimated in our primary care cohort. However, our NAFLD cohort has very similar patient characteristics (Caucasian, obese, middle-aged, with abnormal LFTs) to those reported by Angulo et al (Angulo et al.,

2007), and in many countries the distinction between primary and secondary care is not as clear. The NFS was chosen over other non-invasive scoring systems (i.e. BARD (Harrison et al., 2008); AST-to-platelet ratio index (Wai et al., 2003); FIB-4 (Vallet-Pichard et al., 2006)) and specialised blood tests (i.e. ELF) test (Parkes et al., 2010); Fibrotest (Poynard et al., 2010)) that detect advanced fibrosis for the purpose of our study as it is an easily applicable tool (web-based calculator), has the best reported PPV for scoring systems in secondary care (McPherson et al., 2010), entails minimal extra cost to PCPs (i.e. platelet sampling) and incorporates blood and clinical parameters that are routinely available in primary care. We were not able to validate the NFS against other non-invasive modalities (Castera et al., 2008; Parkes et al., 2010; Poynard et al., 2010) as these had not been developed nor sufficiently studied by the time our study had started. Moreover there are issues about how to validate such modalities in primary care, as it is unlikely and also unethical that liver biopsies would ever be performed in such a large sample of patients or in this setting. NFS is limited to predicting the presence or absence of advanced fibrosis only, and does not distinguish between benign steatosis alone (non-NASH) and the inflammatory process of NASH. Previous studies have highlighted that NAFLD patients with NASH (independent of fibrosis) have a higher risk of death from liver disease and to a greater extent cardiovascular disease than those with non-NASH (Ekstedt et al., 2006; Rafiq et al., 2009). At present, however, non-invasive tools do not exist in primary care to identify individuals with NASH +/- early fibrosis.

Despite a thorough non-invasive aetiology screen and detailed alcohol history 45% had unexplained abnormal LFTs in our cohort. However, as we targeted patients in primary care

that have incidental abnormal LFTs in the absence of a clinical suspicion of underlying liver disease this is not a surprise. Furthermore, unlike previous general population studies (Clark et al., 2003; Bedogni et al., 2005) that only utilised ALT, AST and/or GGT, our study recruited patients with a wider spectrum of LFT analytes to reflect common practice in primary care. It is therefore possible that some of the unexplained abnormal LFTs represent transient (viral) illness, Gilbert's syndrome, under (self-) reported use of alcohol/over-counter medications or non-liver related disease (i.e. bone, muscle) (Clark et al., 2003). The finding that 20% of the unexplained group normalised LFTs within an average of 30 days of re-testing supports this hypothesis. Although USS is the most readily available imaging tool available in primary care, the fact that 18% of the 'unexplained' group had co-existing obesity with diabetes and/or hypertension raises the likely possibility that reliance on ultrasound alone will miss a proportion of cases of NAFLD. The difficulty in detecting the presence of fatty liver with USS is well reported in the morbidly obese and when the degree of fat infiltration is less than 33% of the hepatic content (Saadeh et al., 2002). Furthermore, biopsy reports have shown that fat content is lost towards the more advanced stages of NAFLD, with the resultant fibrotic tissue being undetectable on USS (Adams et al., 2005c). The lack of markers of insulin sensitivity and lipid profile in the study meant we were unable to non-invasively quantify hepatic fat (Kotronen et al., 2009), and hence potentially determine the numbers of undetected NAFLD on USS within the 'unexplained' group.

Our findings have important clinical and public health implications. This study raises awareness that NAFLD accounts for a significant proportion of incidental abnormal LFTs commonly encountered by PCPs, in the absence of a clinical suspicion of liver disease. We

have identified a potential sub-set of NAFLD patients with advanced fibrosis (7.6%) that require early assessment and management in secondary care. We would advocate a certain degree of reassurance with regards to the absence of underlying advanced fibrosis/cirrhosis and an impetus for regular metabolic disease risk assessment and lifestyle modifications in patients with a low NFS (57.2%). In the absence of validated scoring systems, patients at present with an indeterminate NFS require closer surveillance in primary care with referral to secondary care as deemed appropriate by the PCP.

In conclusion, we provide novel information on the severity of NAFLD in a primary care setting, as well as guidance on the triaging of such patients for further investigation and management.

## **CHAPTER 3: SAFETY AND EFFICACY OF LIRAGLUTIDE IN TYPE 2 DIABETIC PATIENTS WITH ELEVATED LIVER ENZYMES: INDIVIDUAL PATIENT DATA META-ANALYSIS OF THE LEAD PROGRAM**

### **3.1 Introduction**

NAFLD and steatohepatitis are common complications in type 2 diabetes, and leading causes of liver disease worldwide. As a result of the alarming growth of type 2 diabetes and central obesity, NAFLD is expected to become a major cause of liver-related mortality and liver transplantation over the next 5 years. Despite this, there are currently no approved therapies of proven benefit for NAFLD in patients with type 2 diabetes (Sanyal et al., 2011).

GLP-1 is an incretin hormone with a potent blood glucose-lowering action mediated via its ability to induce insulin secretion and reduce glucagon secretion in a glucose-dependent manner. Furthermore, GLP-1 slows gastrointestinal motility and increases satiety with reduced food intake (Baggio and Drucker, 2007). Human GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase-4 and other endopeptidases, resulting in a short half-life of 1.5 to 2.0 minutes (Deacon et al., 1995). To overcome this, GLP-1 receptor agonists based on exendin-4 (exenatide) or human analogues (liraglutide), resistant to dipeptidyl peptidase-4, have recently been developed. Liraglutide has been produced using human recombinant DNA technology and shares 97% amino acid sequence homology with native human GLP-1 (Knudsen et al., 2000). The resultant once-daily subcutaneous administration has recently

been licensed in America and Europe for use in type 2 diabetes. Twenty-six weeks liraglutide therapy has been shown to reduce HbA1c by 1.0–1.5%, systolic blood pressure by 2–7 mmHg and weight by 2–3 kg in over 4000 patients with type 2 diabetes studied in the LEAD trials programme (Buse et al., 2009; Garber et al., 2009; Marre et al., 2009; Nauck et al., 2009; Russell-Jones et al., 2009; Zinman et al., 2009). In addition, the GLP-1R agonists, exendin-4 and liraglutide, have been shown to improve liver enzymes, oxidative stress and hepatic steatosis in murine models (Ding et al., 2006; Ben-Shlomo et al., 2011; Mells et al., 2012). *In vitro* data suggest that GLP-1R agonists can act directly on human hepatocytes via a G-protein coupled receptor (Gupta et al., 2010; Svegliati-Baroni et al., 2011) and protect hepatocytes from fatty acid related death (Sharma et al., 2011). These actions suggest that by direct or indirect metabolic mechanisms, liraglutide may be a promising option for the treatment of NAFLD.

To date, human studies investigating the effect of GLP-1R agonists on the human liver have been limited to case reports (Tushuizen et al., 2006; Ellrichmann et al., 2009), solitary case series (Kenny et al., 2010) and uncontrolled open-label retrospective studies (Buse et al., 2007). In light of this limited experience in patients with hepatic injury the US FDA and the European Medicines Agency (EMA) caution against the use of GLP-1 analogues in patients with mild, moderate and severe liver injury. The efficacy and safety of this group of drugs in liver disease, therefore, remains unproven. Therefore, in 2009 I approached Novo Nordisk A/S Ltd with a project proposal to retrospectively obtain unpublished liver data from their LEAD program, in order to investigate the efficacy and safety, prior to designing our own prospective clinical trial. Subsequently, our meta-analysis of individual patient-level data

combined from the six phase III, multi-national, RCTs that comprise the LEAD program, was performed to assess the safety and efficacy of liraglutide on liver parameters in comparison to an active-placebo control. In addition, a sub-study of the LEAD-2 trial was analysed to assess the effect of liraglutide on hepatic steatosis.

## **3.2 Methods**

### **3.2.1 Study population and design**

All subjects from the LEAD clinical development program randomized to 0.6mg, 1.2mg, 1.8 mg/day liraglutide, oral anti-diabetic drugs (OADs) or active/placebo, were included in this meta-analysis. The study design of the six phase III RCTs that comprise the LEAD program are summarized in Table 3-1 (Buse et al., 2009; Garber et al., 2009; Marre et al., 2009; Nauck et al., 2009; Russell-Jones et al., 2009; Zinman et al., 2009). In total, 4456 patients from 40 countries with type 2 diabetes who were unable to maintain glycaemic control ( $\text{HbA1c} \geq 7\%$ ) with diet and exercise alone, or with oral anti-diabetic treatment, were recruited to the LEAD program. This was designed primarily to compare liraglutide alone (or in combination with various OAD) to anti-hyperglycaemic therapies commonly used in type 2 diabetes. The original primary outcome of all the LEAD studies was change in HbA1c from baseline, with secondary outcomes including changes in fasting plasma glucose and weight. Exclusion criteria for the 6 individual LEAD trials included treatment with systemic corticosteroids, liver-specific symptoms and ALT  $\geq 2.5$  times upper limit of normal range of the standard laboratory, renal dysfunction (defined as  $\geq 135 \mu\text{mol/L}$  in males,  $\geq 110 \mu\text{mol/L}$  in females), cancer (except basal or squamous cell skin cancer), and sero-positivity for HBV or HCV. A detailed alcohol history was not taken, although patients with a prior diagnosis of alcohol related liver disease were excluded. Liraglutide (or active-placebo) was injected once daily with a prefilled pen injection device for 26 weeks (52 weeks in LEAD-3) duration. In all studies, the starting dose was liraglutide 0.6 mg/day, titrated after 7 days to 1.2mg. In



studies in which liraglutide 1.8mg was evaluated, subjects were titrated to this dose after a further 7 days at 1.2mg.

LEAD-2 was a 26 week, multi-centre, randomised, double-blinded, active-placebo control, phase III (Nauck et al., 2009). Subjects were randomized to 0.6, 1.2 or 1.8mg/day liraglutide, 4mg/day glimepiride or placebo, all in combination with metformin. LEAD-2 incorporated an optional sub-study to assess the efficacy of 26 weeks treatment with liraglutide on hepatic steatosis and body fat composition. Inclusion criteria and treatment regimen for the sub-study were identical to the main trial.

LEAD Trial	Duration/ Type	N	Baseline data			Main Treatment	Main Comparator	Placebo-control
			Weight (Kg)	HbA <sub>1c</sub> (%)	Previous OAD			
<b>1</b>	26-weeks/ double-blind RCT	1018	81.6	8.4	SU	Lira 0.6 mg Lira 1.2 mg Lira 1.8 mg	Rosiglitazone	Yes
<b>2</b>	26-weeks/ double-blind RCT	1091	88.6	8.4	Metformin	Lira 0.6 mg Lira 1.2 mg Lira 1.8 mg	Glimepiride	Yes
<b>3</b>	52-wk/ double-blind RCT	746	92.6	8.2	none	Lira 1.2 mg Lira 1.8 mg	Glimepiride	No
<b>4</b>	26-weeks/ double-blind RCT	533	97.0	8.5	Metformin + TZD	Lira 1.2 mg Lira 1.8 mg	Placebo	Yes
<b>5</b>	26-weeks/ double-blind RCT*	581	85.4	8.2	Metformin + SU	Lira 1.8 mg	Insulin glargine	Yes
<b>6</b>	26-weeks/ open-label RCT	464	93.0	8.2	Metformin and/or SU	Lira 1.8 mg	Exenatide	No

**Table 3-1. LEAD program design and overview**

\*Only liraglutide and active-placebo treatment arms were double-blinded. Key: RCT, randomised-control trial; SU, sulphonylurea; TZD, thiazolidinedione.

### 3.2.2 Data collection and definitions

Baseline demographics and clinical/laboratory measures were recorded at randomization. The metabolic syndrome was defined as  $\geq 3$  metabolic components (NCEP, 2002). For purposes of this retrospective analysis serum ALT was used as a surrogate marker to estimate the proportion of LEAD participants with liver injury. The new ALT cut-offs as recommended by Prati et al ( $> 30$  IU/L in males,  $>19$  IU/L females) were used to define abnormality (Prati et al., 2002). The NFS (Angulo et al., 2007), a well-validated scoring system, was retrospectively calculated in all subjects at baseline in an attempt to estimate the presence or absence of advanced liver fibrosis in the LEAD program (Angulo et al., 2007). The low cut-off score ( $< -1.455$ ) has a negative predictive value of 88 to 93% and the high cut-off score ( $> +0.676$ ) has a positive predictive value of 79 to 90% for the presence of advanced fibrosis in NAFLD in secondary care populations (Angulo et al., 2007; Wong et al., 2008).

In the LEAD-2 sub-study (Jendle et al., 2009), hepatic steatosis was measured at randomization and 26 weeks using single-slice, non-contrast enhanced abdominal CT. Directly comparing the CT attenuation of the liver to the spleen (internal control) to provide a liver to spleen attenuation ratio (LSAR) has previously been shown to be a valid tool for measuring the presence and severity of hepatic steatosis (Oliva et al., 2006; McKimmie et al., 2008). In this study, the presence of hepatic steatosis was defined as an LSAR  $< 1.0$  (McKimmie et al., 2008), with increases in the ratio indicating reductions in steatosis (Oliva et al., 2006).

### **3.2.3 Safety Profile**

Individual patient-level data of trial withdrawal, treatment-emergent AEs and serious AE (SAE) were combined from the LEAD program to enable a descriptive comparison of the safety profile of 26 weeks treatment with liraglutide (1.2mg/1.8mg) between patients with normal and abnormal baseline ALT. A treatment-emergent AE was defined as an event occurring between the first and last dose (plus 7 days) or starting before first dose with increasing severity during treatment. All treatment emergent AEs with an incidence of 10% or more in any pooled treatment group (active-placebo, liraglutide 1.2mg, liraglutide 1.8mg) organized by system organ class and preferred term are reported.

### **3.2.4 Statistical Analysis**

The six phase III RCTs were combined to facilitate an individual patient-level data meta-analysis of liraglutide versus active-placebo control. The analysis was based on an intention-to-treat (ITT) population (4442 out of 4456 recruited), defined as subjects from each of the individual trials who were randomized and exposed to at least one dose of study treatment (0.6mg, 1.2 mg, 1.8 mg liraglutide, active-placebo control or other anti-diabetic treatments). Descriptive statistics were applied to characterize the whole ITT study cohort and the individual treatment groups. All continuous clinical and laboratory variables are reported as means (standard deviation [SD]) and categorical variables as numbers (percentages), unless stated. The significance level was set at  $p < 0.05$ .

#### *3.2.4.1 Changes in Liver Enzymes – meta-analysis*

Change in ALT after 26 weeks treatment was analyzed using an analysis of covariance (ANCOVA) model with the trial, country, randomized treatment, previous anti-diabetic medication, normality of ALT at baseline, and the interaction effect between randomized treatment and the normality of ALT at baseline as fixed effects. This analysis, therefore, allowed for adjustment of potentially confounding factors, including concomitant oral anti-diabetic medications. Post-baseline data were imputed using the last observation carried forward (LOCF) method in case of missing observations at week 26. The ANCOVA model was then repeated for subjects with abnormal baseline ALT, where changes in weight and/or changes in HbA<sub>1c</sub> were included as covariates to investigate if the effect of 26 weeks liraglutide (1.8mg/day) treatment (*versus* active-placebo) on ALT was independent of its effects on weight and/or glycaemic control.

#### *3.2.4.2 Changes in Hepatic Steatosis – LEAD-2 sub-study only*

Change in CT-measured hepatic steatosis (i.e. LSAR) after 26 weeks treatment in the LEAD-2 sub-study were analyzed using a repeated measures model with previous anti-diabetic treatment, country and randomized treatment as fixed effects, and baseline values as covariates. Furthermore, interaction terms of previous anti-diabetic treatment by visit, country by visit, randomized treatment by visit and baseline values by visit were included, and an unstructured covariance matrix for parameter of interest (LSAR) within the same subject was employed. The analysis was based on the ITT population in LEAD-2 to estimate

change from baseline and comparison between liraglutide and active-placebo. The repeated measures model with actual values (rather than ANCOVA with LOCF) was selected in order to reduce bias in treatment effect and residual SD estimates due to the relatively small numbers in each treatment arm. The repeated measures model was repeated with changes in weight and/or changes in HbA<sub>1c</sub> set as covariates to investigate if the effect of 26 weeks treatment with liraglutide 1.8mg (*versus* active-placebo) on LSAR was independent of its effects on weight and/or glycaemic control.

### 3.3 Results

#### 3.3.1 Baseline demographics – meta-analysis

4442 patients were included in the ITT individual patient-level data meta-analysis of the LEAD program, of which 2734 (61.5%) received liraglutide and 524 (11.8%) received active-placebo to enable comparison. The remaining 1184 (26.7%) were randomised to other anti-diabetic medications (analysed, but data not presented). The baseline demographics and clinical characteristics were similar amongst the patients who received either liraglutide (n=2734) or active-placebo (n=524) injections (Table 3-2). The mean age was 55.9 (SD 10.1) years with predominance towards Caucasian race (78.6%). 62% of the cohort had the metabolic syndrome at baseline with a mean BMI of 31.5 (SD 5.4) kg/m<sup>2</sup> and poor glycaemic control (HbA1c mean 8.3% [SD 1.0]).

50.8% (2241/4415; 27 missing data) patients had an abnormal ALT at baseline, with mean values of 33.8 (SD 14.9) IU/L in females and 47.3 (18.3) IU/L in males. A high NFS (>+0.676) was found in 6.3% (266/4238; mean score 1.13) of patients suggesting the presence of advanced liver fibrosis (Stages F3/F4 on Kleiner classification (Kleiner et al., 2005)). The presence of advanced liver fibrosis, however, could not be confidently excluded in 61.7% (2613/4238) of the diabetic patients who scored an indeterminate value with the NFS (-1.455 to +0.676). Advanced fibrosis was predicted to be absent in 32.1% (1359/4238) subjects with a low NFS (<-1.455).

	<b>LEAD program Total</b>	<b>Liraglutide <i>versus</i> Placebo</b>				
		<b>Total</b>	<b>Placebo</b>	<b>Lira 0.6 mg</b>	<b>Lira 1.2 mg</b>	<b>Lira 1.8 mg</b>
ITT, N [% of randomised]	<b>4442 [99.7]</b>	3258 [99.7]	524 [99.2]	475 [100]	896 [99.8]	1363 [99.8]
Male Sex, N [%]	<b>2378 [53.5]</b>	1732 [53.2]	288 [55.0]	277 [58.3]	449 [50.1]	718 [52.7]
Age (years)	<b>55.9 [10.1]</b>	55.7 [10.1]	55.7 [9.8]	55.9 [10.2]	55.9 [10.0]	55.6 [10.2]
Ethnicity, N [%]						
White	<b>3493 [78.6]</b>	2556 [78.5]	419 [80.0]	356 [74.9]	697 [77.8]	1084 [79.5]
Asian/Hawaiian/Pacific	<b>565 [12.7]</b>	417 [12.8]	61 [11.6]	103 [21.7]	104 [11.6]	149 [10.9]
Black/African American	<b>257 [5.8]</b>	198 [6.1]	27 [5.2]	11 [2.3]	76 [8.5]	84 [6.2]
American Indian/Alaskan	<b>5 [0.1]</b>	4 [0.1]	2 [0.4]	0 [0.0]	1 [0.1]	1 [0.1]
Other	<b>94 [2.1]</b>	67 [2.1]	10 [1.9]	5 [1.1]	18 [2.0]	34 [2.5]
Previous OAD, N [%]						
Combination	<b>2703 [60.9]</b>	2025 [62.2]	408 [77.9]	323 [68.0]	455 [50.8]	839 [61.6]
Monotherapy	<b>1467 [33.0]</b>	1055 [32.4]	116 [22.1]	152 [32.0]	350 [39.1]	437 [32.1]
Diet only	<b>272 [6.1]</b>	178 [5.5]	0 [0.0]	0 [0.0]	91 [10.2]	87 [6.4]
<b>Metabolic Parameters</b>						
Metabolic Syn.*, N [%]	<b>2754 [62.0]</b>	2011 [61.7]	324 [61.8]	275 [57.9]	570 [63.6]	842 [61.8]
Systolic BP (mmHg)	<b>131.3 [15.2]</b>	130.9 [15.0]	131.5 [15.0]	131.1 [14.8]	130.4 [14.8]	130.9 [15.2]
Weight (kg)	<b>88.6 [18.9]</b>	88.7 [19.0]	90.3 [18.5]	85.2 [17.6]	88.7 [19.2]	89.4 [19.4]
BMI (kg/m <sup>2</sup> )	<b>31.5 [5.4]</b>	31.5 [5.4]	31.9 [5.2]	30.3 [4.9]	31.7 [5.4]	31.7 [5.5]
Male waist circum. (cm)	<b>106.9 [13.4]</b>	107.1 [13.5]	108.0 [13.2]	104.9 [11.9]	107.8 [13.7]	107.0 [14.1]
Female waist circum. (cm)	<b>102.4 [13.6]</b>	102.4 [13.5]	104.0 [12.9]	98.1 [12.8]	102.3 [13.6]	103.2 [13.7]
Cholesterol (mmol/L)	<b>4.9 [1.2]</b>	4.9 [1.2]	5.0 [1.2]	4.9 [1.1]	5.0 [1.2]	4.9 [1.2]
Triglycerides (mmol/L)	<b>2.3 [1.9]</b>	2.3 [1.8]	2.5 [2.4]	2.2 [1.4]	2.2 [1.6]	2.2 [1.8]
FPG (mmol/L)	<b>9.7 [2.4]</b>	9.8 [2.4]	9.8 [2.3]	10.1 [2.4]	9.8 [2.5]	9.7 [2.4]
HbA1c (%)	<b>8.3 [1.0]</b>	8.4 [1.0]	8.4 [1.0]	8.4 [1.0]	8.4 [1.1]	8.3 [1.0]
Duration of Diabetes (yrs)	<b>7.7 [5.7]</b>	7.7 [5.6]	8.6 [5.9]	7.3 [4.9]	7.1 [5.5]	7.9 [5.7]
<b>Liver Enzymes (IU/L)</b>						
<b>Total ALT</b>						
N	<b>4415</b>	3235	517	473	889	1356
Mean [SD]	<b>29.4 [16.6]</b>	28.9 [16.1]	28.2 [15.7]	29.0 [15.8]	28.5 [15.5]	29.5 [16.7]
<b>Normal ALT**</b>						
N (% ITT)	<b>2174 [49.2]</b>	1627 [50.3]	269 [52.0]	252 [53.3]	449 [50.5]	657 [48.5]
Mean (SD)	<b>19.1 [5.6]</b>	18.9 [5.6]	18.4 [5.3]	19.4 [5.6]	19.2 [5.8]	18.7 [5.6]
<b>Abnormal ALT</b>						
N (% ITT)	<b>2241 [50.8]</b>	1608 [49.7]	248 [48.0]	221 [46.7]	440 [49.5]	669 [51.5]
Mean (SD)	<b>39.4 [17.7]</b>	39.1 [16.8]	38.8 [16.3]	40.0 [16.5]	38.0 [16.5]	39.6 [17.3]

**Table 3-2. Baseline Demographics and Clinical Parameters of LEAD 1-6 trials: Intention-to-treat (ITT) cohort.**

Values are mean [SD] unless stated otherwise. Percentages include missing values.

\* ≥3 components of the metabolic syndrome (ATP III criteria (NCEP, 2002)).

\*\* Normal reference range for ALT ≤19 IU/L females; ≤30 IU/L males (Prati et al., 2002).

### **3.3.2 Safety Profile – meta-analysis**

The frequency of adverse events or subsequent withdrawal rates from liraglutide (1.2/1.8mg) was similar between patients with or without abnormal ALT at baseline (Table 3-3). The incidence of gastrointestinal and hepatobiliary SAEs with liraglutide 1.2 mg or 1.8mg was comparable in patients with abnormal baseline ALT (1.2mg, 1.1%; 1.8mg, 0.6%) and with normal ALT at baseline (1.2mg, 1.1%; 1.8mg, 0.9%).

### **3.3.3 Change in ALT – meta-analysis**

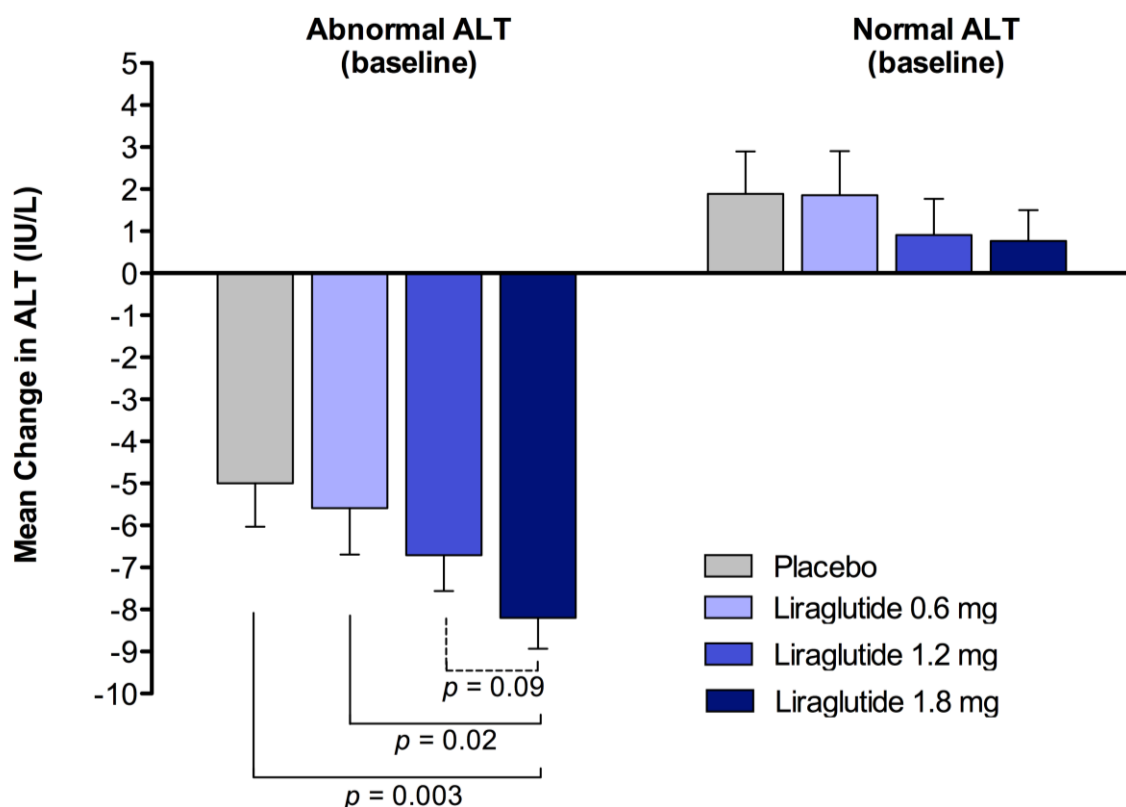
26 weeks treatment with liraglutide 1.8mg significantly reduced ALT in patients with abnormal baseline readings in comparison to active-placebo (-8.20 vs. -5.01 IU/L;  $p = 0.003$ ) (Figure 3-1). This effect was dose-dependent with greater reductions in ALT seen with the 1.8mg dose than the 1.2mg dose (1.8 vs. 1.2mg difference, -1.49 IU/L,  $p = 0.09$ ) and 0.6mg daily (1.8 vs. 0.6mg, - 2.61 IU/L;  $p = 0.02$ ). The improvements in ALT with liraglutide 1.8mg versus active-placebo were eliminated on correcting for change in weight (corrected mean difference vs. placebo, -1.41 IU/L;  $p = 0.21$ ) and in HbA<sub>1c</sub> (corrected mean difference vs. placebo, 0.57 IU/L;  $p = 0.63$ ). No significant differences in ALT were seen between placebo and the lower doses of liraglutide (0.6mg/1.2mg).



	Patients with normal ALT at baseline, N (%)			Patients with abnormal ALT at baseline, N (%)		
	Placebo	Lira 1.2mg	Lira 1.8mg	Placebo	Lira 1.2mg	Lira 1.8mg
<b>Safety Population</b>	269 (100)	449 (100)	659 (100)	248 (100)	440 (100)	699 (100)
<b>Overall withdrawal rate</b>	74 (27.5)	96 (21.4)	133 (20.2)	73 (29.4)	89 (20.2)	111 (15.9)
<b>Participants with any SAE</b>	18 (6.7)	35 (7.8)	43 (6.5)	11 (4.4)	29 (6.6)	32 (4.6)
<b>Participants with AE</b>	178 (66.2)	358 (79.7)	495 (75.1)	163 (65.7)	353 (80.2)	547 (78.3)
<b>Withdrawal due to AE</b>	7 (2.6)	35 (7.8)	76 (11.5)	6 (2.4)	42 (9.5)	50 (7.2)
<b>Participants with SAE, by system organ class</b>						
<b>Gastrointestinal disorders</b>	0 (0.0)	4 (0.9)	5 (0.8)	1 (0.4)	4 (0.9)	3 (0.4)
<b>Hepatobiliary disorders</b>	0 (0.0)	1 (0.2)	1 (0.2)	1 (0.4)	1 (0.2)	2 (0.3)
<b>Cardiac disorders</b>	3 (1.1)	7 (1.6)	9 (1.4)	2 (0.8)	6 (1.4)	5 (0.7)
<b>Infections and infestations</b>	5 (1.9)	6 (1.3)	6 (0.9)	1 (0.4)	5 (1.1)	5 (0.7)
<b>Metabolism and nutrition disorders</b>	1 (0.4)	1 (0.2)	2 (0.3)	1 (0.4)	0 (0.0)	1 (0.1)
<b>Neoplasms (benign, malignant and unspecified)</b>	3 (1.1)	3 (0.7)	6 (0.9)	0 (0.0)	5 (1.1)	9 (1.3)
<b>Renal and urinary disorders</b>	1 (0.4)	1 (0.2)	1 (0.2)	0 (0.0)	0 (0.0)	0 (0.0)
<b>Respiratory, thoracic and mediastinal disorders</b>	1 (0.4)	0 (0.0)	1 (0.2)	0 (0.0)	2 (0.5)	1 (0.1)
<b>Nervous system disorders</b>	1 (0.4)	4 (0.9)	6 (0.9)	1 (0.4)	3 (0.7)	1 (0.1)
<b>Musculoskeletal and connective tissue disorders</b>	1 (0.4)	3 (0.7)	7 (1.1)	1 (0.4)	2 (0.5)	1 (0.1)
<b>Investigations</b>	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.4)	0 (0.0)	0 (0.0)
<b>Endocrine disorders</b>	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (1.1)	3 (0.4)
<b>General/Other*</b>	6 (2.2)	7 (1.6)	6 (0.9)	3 (1.2)	6 (1.4)	6 (0.9)
<b>AE with an incidence ≥10% in any treatment group, by system organ class/preferred term</b>						
<b>Gastrointestinal disorders</b>	47 (17.5)	207 (46.1)	286 (43.4)	45 (18.1)	199 (45.2)	325 (46.5)
- Diarrhoea	11 (4.1)	52 (11.6)	70 (10.6)	12 (4.8)	55 (12.5)	118 (16.9)
- Nausea	12 (4.5)	103 (22.9)	141 (21.4)	14 (5.6)	86 (19.5)	164 (23.5)
<b>General disorders, injection site conditions</b>	26 (9.7)	68 (15.1)	77 (11.7)	20 (8.1)	65 (14.8)	85 (12.2)
<b>Infections and infestations</b>	92 (34.2)	159 (35.4)	215 (32.6)	83 (33.5)	194 (44.1)	263 (37.6)
- Upper resp. tract infection	22 (8.2)	38 (8.5)	40 (6.1)	11 (4.4)	45 (10.2)	53 (7.6)
<b>Investigations</b>	25 (9.3)	45 (10.0)	44 (6.7)	15 (6.0)	29 (6.6)	56 (8.0)
<b>Metabolism and nutrition disorders</b>	17 (6.3)	78 (17.4)	94 (14.3)	22 (8.9)	66 (15.0)	86 (12.3)
<b>Musculoskeletal and connective tissue disorders</b>	43 (16.0)	80 (17.8)	104 (15.8)	27 (10.9)	88 (20.0)	123 (17.6)
<b>Nervous system disorders</b>	33 (12.3)	87 (19.4)	120 (18.2)	33 (13.3)	102 (23.2)	138 (19.7)
- Headache	16 (5.9)	36 (8.0)	59 (9.0)	23 (9.3)	61 (13.9)	76 (10.9)

**Table 3-3. Safety profile of 1.2 and 1.8 mg Liraglutide in patients with normal and abnormal liver enzymes (ALT) in LEAD 1-6 trials.**

\* General/Other includes: Congenital, familial and genetic disorders; eye disorders; General disorders and administration site conditions; injury (i.e. fracture), poisoning and procedural complications (i.e. surgery); psychiatric disorders; reproductive system; skin disorders; and vascular disorders.

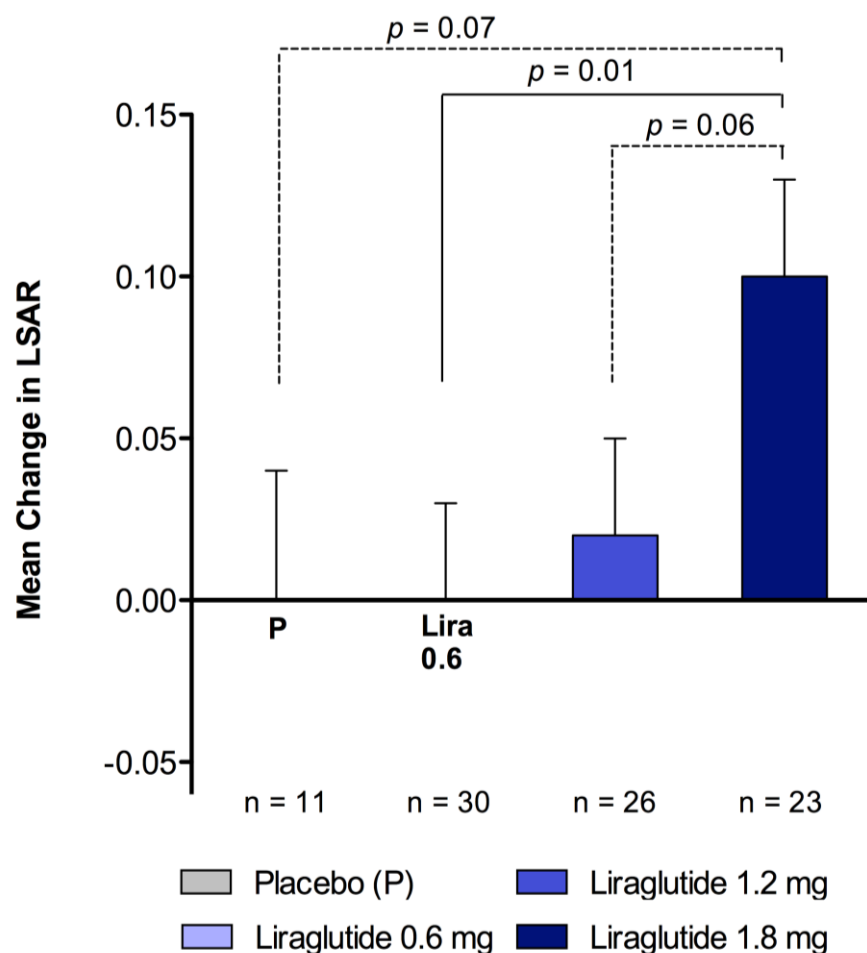


**Figure 3-1. Changes in ALT with 26-weeks treatment of liraglutide versus placebo in type 2 diabetes patients with abnormal (left) and normal (right) ALT at baseline.**  
Meta-analysis of LEAD-1 to LEAD-6.

### 3.3.4 Change in Hepatic Steatosis – LEAD-2 sub-study

In LEAD-2, the presence of hepatic steatosis was confirmed on CT imaging in 64.4% (96/149) of individuals at baseline, of which 58.3% (56/96) had at least 30% hepatic steatosis on CT (LSAR <0.8 (Park et al., 2006)). In keeping with the ALT data (above), the effect of liraglutide on LSAR appeared to be dose-dependent (Figure 3-2). At 26 weeks there was a trend towards an improvement in LSAR with liraglutide 1.8mg (n=23) compared to the 11 patients on active-placebo (mean difference +0.10 [95% CI -0.01 to +0.20]; p = 0.07). This difference at 26 weeks was reduced when correcting for changes in weight (mean difference +0.06

[95% CI -0.04 to +0.15];  $p = 0.25$ ) and  $\text{HbA}_{1c}$  (mean difference 0.00 [95% CI -0.11 to +0.10];  $p = 0.93$ ). No significant differences in LSAR were seen between placebo and the lower doses of liraglutide 0.6mg (mean difference 0.00 [95% CI -0.11 to +0.10];  $p = 0.90$ ) and 1.2mg (mean difference 0.02 [95% CI -0.09 to +0.12];  $p = 0.73$ ).



**Figure 3-2. Changes in hepatic steatosis with 26-weeks treatment of liraglutide versus placebo in patients with type 2 diabetes.**

Analysis of LEAD-2. Data for glimepiride not reported here. Changes in hepatic steatosis represented by changes in CT measured liver:spleen attenuation ratio (LSAR).

### 3.4 Discussion

This individual patient-level data meta-analysis of the LEAD program demonstrates that 26 weeks treatment with liraglutide 1.8mg/day is well-tolerated, safe to use and results in significant improvements in liver enzymes in patients with type 2 diabetes and asymptomatic liver injury. The efficacy of 26 weeks liraglutide on liver enzymes is dependent on drug dosage and appears to be mediated by its effect on weight change and glycaemic control. Gastrointestinal symptoms, namely nausea and diarrhoea, are the commonest adverse events associated with liraglutide, but are mainly transient in nature (< 2 weeks) and occur no more frequently in patients with abnormal liver enzymes.

Half of the patients in the meta-analysis had abnormal liver transaminases at baseline in keeping with previous diabetes trials with comparable patient characteristics (Buse et al., 2007). Furthermore, almost two-thirds (64.4%) of subjects in the LEAD-2 sub-study had hepatic steatosis confirmed on CT. Although H-MRS is the recognised non-invasive 'gold-standard' for quantifying hepatic steatosis (Bohte et al., 2011), our figures for steatosis are in agreement with rates (56.9% to 69.5%) previously reported in large cross-sectional studies that utilised ultrasound and/or MRS (Targher et al., 2007; Williamson et al., 2011). Entry criteria to the LEAD programme included negative HBV/HCV serology and no history of steroid-use and/or alcoholic liver disease, leading us to conclude that the majority of these cases are likely due to NAFLD. This view is further supported by the compelling data that exists linking NAFLD to type 2 diabetes, obesity and the metabolic syndrome (Williamson et al., 2011; Wong et al., 2012a), all of which were prevalent in our study population. However,

due to the lack of a detailed alcohol consumption history and serology for rarer liver conditions (such as autoimmune disease and haemochromatosis), other causes of liver damage in our study population cannot be categorically excluded.

Clinical trials in NAFLD demonstrate that reductions in ALT correlate with histological improvements in liver inflammation (Suzuki et al., 2006; Aithal et al., 2008; Ratzliff et al., 2008; Sanyal et al., 2010). Here, we report significant improvements in the liver injury biomarker ALT with 26 weeks of liraglutide 1.8mg (-8.20 IU/L from a baseline mean of 39.6 IU/L) in comparison to an active-placebo control. Previous studies with GLP-1R agonists, namely exenatide, have demonstrated a similar magnitude of reduction in ALT from baseline, but lack comparison to a placebo control (Buse et al., 2007). Although significant, the effects of 26-weeks liraglutide 1.8mg on ALT seen in our study may have been diluted by the placebo effect. Placebo effects have previously been reported to be as high as 19-30% in prospective NAFLD trials (Promrat et al., 2010; Sanyal et al., 2010), which is of little surprise given the established efficacy of lifestyle modification in such metabolic conditions (Thoma et al., 2012).

Our study highlights a trend towards improvements in hepatic steatosis with liraglutide 1.8mg in comparison to placebo controls over 26-weeks ( $p=0.07$ ). This, together with the significant baseline changes in hepatic steatosis with liraglutide 1.8mg reinforces findings from a previous case-report (Tushuizen et al., 2006) and a case-series investigating the histological effects of exenatide in eight patients with type 2 diabetes (Kenny et al., 2010). The latter group reported decreased NASH activity (defined as reduced hepatocyte

ballooning and inflammation and/or steatosis) in 3 out of the 8 type 2 diabetic patients receiving 28-weeks exenatide (Kenny et al., 2010).

Our data suggest that the effect of 26 weeks liraglutide on weight loss and to a similar extent its effect on glycaemic control are the main factors in significantly reducing ALT in comparison to active-placebo controls. Although these effects appear to be correlated to liraglutide's benefit on weight and HbA1c, in the absence of a prospective study powered specifically for liver end-points, we are not able to rule out the possibility of a direct effect of liraglutide on liver injury. There are increasing murine and *in vitro* data to support a direct mechanism of GLP-1R agonists on the liver, over and above its role as an incretin hormone (Gupta et al., 2010; Ben-Shlomo et al., 2011; Svegliati-Baroni et al., 2011; Mells et al., 2012). Not only has the GLP-1R been identified on both murine and human hepatocytes (Ding et al., 2006; Gupta et al., 2010; Svegliati-Baroni et al., 2011), but GLP-1R agonist treatment in cell culture decreases triglyceride and free fatty acid stores in the absence of insulin (Gupta et al., 2010; Mells et al., 2012). Furthermore, recent *in vitro* evidence would suggest that GLP-1R agonists markedly improve the ability of the hepatocyte to handle excess free fatty acids and lipid production by modulating lipid transport, beta-oxidation and de novo lipogenesis (Gupta et al., 2010; Ben-Shlomo et al., 2011; Mells et al., 2012), all of which have been implicated in the pathogenesis of NAFLD.

Our study has a number of strengths. First, this is the first individual patient-level data meta-analysis (six large double-blinded RCTs) to focus on the effects of a human GLP-1 analogue on liver parameters in patients with type 2 diabetes, and the first to report comparisons to

an active-placebo control whilst controlling for several confounding factors, including concomitant OAD treatment (i.e. TZDs, metformin). Second, the meta-analysis provides a descriptive overview of the safety profile of liraglutide in type 2 diabetic patients with and without abnormal blood liver enzymes prior to treatment. Even though the long-term adverse events remain unknown, this study provides valuable reassurance in the safe short-term use of liraglutide in the presence of mild to moderate liver injury (ALT > upper limit of normal to < 2.5 times upper limit) and potential steatosis. The main limitation of this study is that the six RCTs combined in this meta-analysis were powered on glycaemic control and not changes in liver parameters. It is important to note that differences in patient populations between the six trials, in terms of previous exposure to anti-diabetic therapy and baseline abnormality of liver enzymes, were included as fixed effects in this analysis. Nevertheless, there may be a degree of heterogeneity (despite similar eligibility criteria) between the six trials included in this meta-analysis. Finally, the lack of liver biopsy precludes the ability to accurately validate the severity of underlying liver injury with regards to the NFS predictions and most importantly, to validate the accuracy of ALT as a serial marker of liver inflammation in our cohort.

In conclusion, our large-scale study highlights that 26 weeks treatment with liraglutide 1.8mg has an acceptable safety profile and significantly improves liver enzymes versus placebo in patients with type 2 diabetes and asymptomatic liver injury. These effects appear to be mediated by the effect of liraglutide on weight loss and glycaemic control. Our data support the rationale to prospectively investigate GLP-1 analogues in liver injury associated with type 2 diabetes and the metabolic syndrome.

## **CHAPTER 4: LIRAGLUTIDE EFFICACY AND ACTION IN NONALCOHOLIC STEATOHEPATITIS (LEAN): STUDY PROTOCOL FOR A PHASE II MULTI-CENTRE, DOUBLE-BLINDED RANDOMISED-CONTROLLED TRIAL**

### **4.1 Introduction**

NAFLD is now the commonest cause of chronic liver disease, affecting up to 30% of the general population (Bellentani et al., 1994; Browning et al., 2004; Armstrong et al., 2012) and 70-90% of high-risk individuals (Bellentani et al., 2004; Browning et al., 2004). This prevalence relates to the dramatic rise in recent years of morbid obesity and type 2 diabetes. Even though simple hepatic steatosis (without fibrosis) is arguably a benign condition, up to a quarter of patients with NAFLD have the more severe, inflammatory condition known as NASH (Williams et al., 2011). Patients with NASH have an increased risk of progression to cirrhosis, liver failure and hepatocellular carcinoma (Bugianesi et al., 2002), and are expected to become the commonest indication for liver transplantation in forthcoming years (Charlton et al., 2011). Despite this, there are no universally accepted pharmacological therapies for NASH. Therefore the need for novel, safe agents in NASH is of paramount importance to prevent disease progression and the accompanying clinical burden.

The strong association of NASH with the metabolic syndrome, in particular central adiposity and insulin resistance, provides strong rationale for investigating therapies that induce



weight loss and insulin sensitivity. The gut-derived incretin hormone, GLP-1 is therefore an attractive target option in NASH. Native GLP-1 has a potent blood glucose-lowering action mediated via its ability to induce insulin secretion and reduce glucagon secretion in a glucose-dependent manner, as well as suppressing appetite and slowing gastric emptying (Baggio and Drucker, 2007). Human GLP-1, however, only has a short half-life (1.5-2.0 mins) as it is rapidly degraded by the enzyme dipeptidyl peptidase-4 (Deacon et al., 1995). Liraglutide (Victoza®) is a long-acting (half-life 13 hours) GLP-1 analogue with 97% structural homology to the native hormone and is administered OD by subcutaneous injection (Knudsen et al., 2000). Liraglutide has been shown to cause dose-dependent weight loss (Astrup et al., 2009; Jendle et al., 2009), decrease HbA1c, systolic blood pressure and improve beta-cell function (Buse et al., 2009; Garber et al., 2009; Marre et al., 2009; Nauck et al., 2009; Russell-Jones et al., 2009; Zinman et al., 2009). Subsequently, it has been licensed for glycaemic control in overweight patients with type diabetes (Mayor, 2010). There is, however, a paucity of data in patients with liver disease, and in particular histological-defined NASH.

GLP-1 analogues, including liraglutide, have been shown to improve liver enzymes, oxidative stress and hepatic steatosis in murine models *in vivo* and in isolated *in vitro* murine and human hepatocyte studies (Ding et al., 2006; Gupta et al., 2010; Ben-Shlomo et al., 2011; Sharma et al., 2011; Svegliati-Baroni et al., 2011; Mells et al., 2012). To date, human studies investigating the effect on liver injury have been limited to case reports (Tushuizen et al., 2006; Ellrichmann et al., 2009), solitary case series (n=8) (Kenny et al., 2010) and retrospective (*liver enzyme*) studies in patients with type 2 diabetes (Buse et al., 2007). Our

large meta-analysis of six phase III RCT, that comprised the LEAD trials program (>4000 patients), highlighted that 26-weeks treatment with 1.8mg OD liraglutide was well-tolerated and resulted in significant improvements in liver enzymes compared to placebo-control in overweight patients with type diabetes (Armstrong et al., 2013d). However, limitations of this study were the retrospective nature of its analysis and the lack of any liver biopsy data.

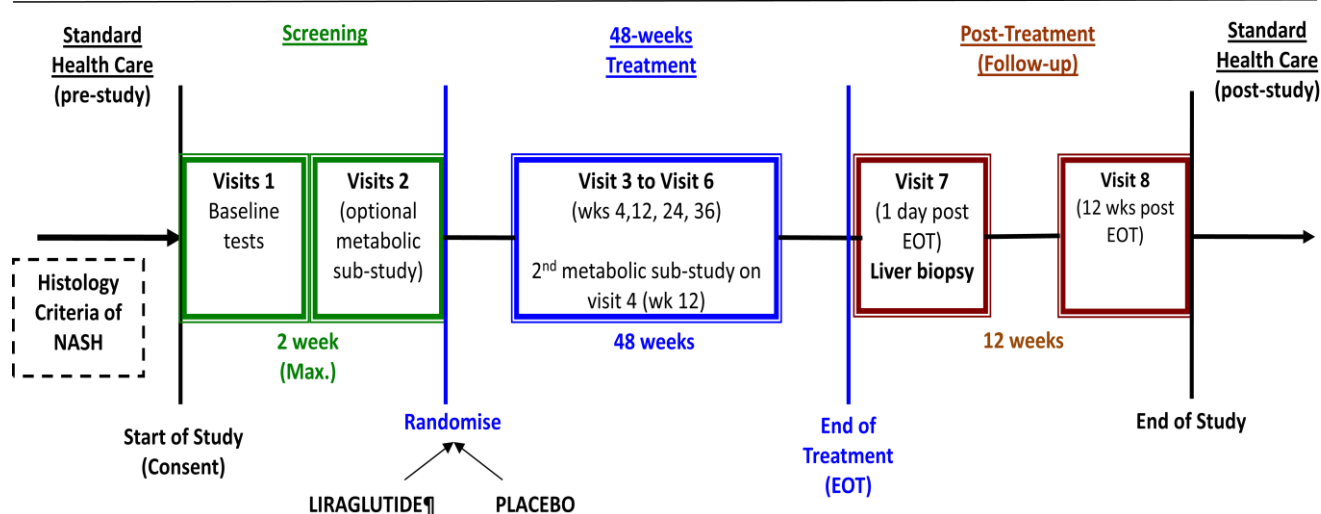
On this basis, we hypothesised that 48 weeks treatment with liraglutide would result in significant improvements in liver histology in overweight patients with NASH. To test this hypothesis, we designed a phase II, multi-centre, double-blinded, placebo-controlled RCT, entitled 'Liraglutide Efficacy and Action in NASH (LEAN).'

## **4.2 Methods**

### **4.2.1 Study Design Overview**

LEAN is a 48 week multi-centre, double-blinded, placebo-controlled randomised clinical trial of treatment with the once daily human GLP-1 analogue, liraglutide (Victoza®), for adults with biopsy-proven NASH. Screening was undertaken within 14 days of randomisation to assess eligibility and collect baseline data. Patients who satisfied the eligibility criteria were randomly assigned (1:1) to receive OD subcutaneous injections of either 1.8 mg liraglutide (experimental) or liraglutide-placebo (control). After which, a 12-week washout period is scheduled.

The primary outcome measure will be assessed using an intention-to-treat analysis of the proportion of evaluable patients achieving an improvement in liver histology between liver biopsies at baseline (within 6 months of screening) and after 48 weeks of treatment. Histological improvement will be defined as a combination of the disappearance of active steatohepatitis (i.e. disappearance of hepatocyte ballooning) and no worsening in fibrosis (Kleiner Fibrosis score (Kleiner et al., 2005)). A schematic of the trial design is summarised in (Figure 4-1).



¶Liraglutide or placebo will be dose titrated from 0.6mg to 1.8mg over the 1<sup>st</sup> 14 days

**Figure 4-1. Schematic of LEAN trial design.**

Eligible participants are randomly assigned to 48 weeks treatment of once-daily (OD) subcutaneous injections (SC) of either 1.8mg liraglutide or placebo-control. Both the trial investigators and the participants are blinded to drug allocation. Key: EOT, end of treatment.

#### 4.2.2 Ethical and regulatory approval

The National Research Ethics Service (NRES) East Midlands – Northampton committee (previously known as Leicestershire, Northamptonshire and Rutland Research Ethics Committee) (UK) and the Medicines and Healthcare products Regulatory Agency (MHRA) approved all versions (inc. current version 7.0) of the study protocol. In addition, all 5 recruitment sites obtained approval from their respective hospital Research and Development (R&D) departments prior to commencing screening.

### 4.2.3 Treatment groups

Patients who satisfied the eligibility criteria were randomly assigned on a 1:1 basis to 48-weeks treatment of either liraglutide (Victoza®; 1.8mg OD) or liraglutide-placebo control (1.8mg OD).

#### 4.2.3.1 Liraglutide (active experimental group)

Liraglutide (Victoza®, Novo Nordisk A/S, Bagsvaerd, Denmark) was supplied in a cartridge contained in a pre-filled multi-dose disposable pen. Each pre-filled pen contained 18 mg liraglutide in 3 ml of clear, colourless, isotonic solution (including water for injections, disodium phosphate dehydrate, propylene glycol and phenol). Liraglutide was administered OD, at any time of the day, as a single subcutaneous injection into the abdomen, thigh or upper arm using the pre-filled pen (30 or 31 gauge needles). Participants were encouraged to inject liraglutide at the same time each day, according to which was the most convenient time for them. Participants were instructed to perform an air shot of 0.2 µl before the first use of each new pre-filled pen to ensure that it functioned correctly.

To improve gastro-intestinal tolerability participants underwent a 14 day dose titration period in keeping with previous reports (Buse et al., 2009; Garber et al., 2009; Marre et al., 2009; Nauck et al., 2009; Russell-Jones et al., 2009; Zinman et al., 2009). The dose was titrated by 0.6 mg every 7 days from a starting dose of 0.6mg OD until the maximum dose of 1.8 mg OD was achieved. Prior to the current trial design, no studies had investigated any

form of GLP-1 based therapy in patients with biopsy-confirmed NASH or any other form of liver disease. Therefore, the rationale for using a dose of 1.8mg OD was based upon previous reports in overweight patients with or without type 2 diabetes (Buse et al., 2009; Garber et al., 2009; Marre et al., 2009; Nauck et al., 2009; Russell-Jones et al., 2009; Zinman et al., 2009). Furthermore, a large meta-analysis of six phase III clinical trials (LEAD program) of liraglutide therapy for poorly controlled type 2 diabetes found that patients with abnormal liver transaminases had a similar drug safety profile to those with normal liver transaminases. In addition, greater improvements in liver transaminases and CT-measured hepatic steatosis were seen with 1.8mg liraglutide than 1.2 and 0.6mg doses (Armstrong et al., 2013d).

#### *4.2.3.2 Liraglutide-placebo (inactive, placebo-control group)*

Liraglutide-placebo (Victoza®, Novo Nordisk A/S, Bagsvaerd, Denmark) was packaged, administered and dose-titrated in an identical manner to the liraglutide comparator, described above. The composition of the placebo solution for injection was identical to its comparator, with the exclusion of the active liraglutide substance. A placebo was used to provide an assessment of the level of response with an injectable placebo, which could potentially be higher than that seen with oral placebo agents.

#### 4.2.3.3 *Concomitant Therapy*

No dose reductions of liraglutide or placebo were allowed throughout the 48 week treatment period. Previous treatment with oral anti-diabetic drugs (metformin and/or sulphonylurea) was continued at the same dose in participants with type 2 diabetes at randomisation. In the event of recurrent major hypoglycaemic episodes (requiring medical or hospital intervention), the dose of the sulphonylurea was reduced by 50% at the discretion of the investigators. The reported rate of hypoglycaemia in the literature, with liraglutide monotherapy or in combination with metformin, is very low (Buse et al., 2009; Garber et al., 2009; Marre et al., 2009; Nauck et al., 2009; Russell-Jones et al., 2009; Zinman et al., 2009). However in the event of recurrent major hypoglycaemic episodes in which no dose reduction could be undertaken (i.e. not on a sulphonylurea) the subject was withdrawn from treatment at the discretion of the chief investigator.

Glycaemic control was assessed at each 12-weekly trial visit with self-measured plasma glucose readings and HbA1c. In the event that glycaemic control deteriorated, defined as HbA1c > 9.0% (75 mmol/mol), the subject was informed and counselled with regards to commencing open-labelled long-acting OD insulin detemir (Levemir®). However, the patient's participation in the trial was not jeopardised if they did not wish to start insulin detemir. The Insulin detemir dose was titrated by trial investigators in accordance with European guidelines ([www.ema.europa.eu](http://www.ema.europa.eu)) to ensure that the subject's standard of diabetes care was not significantly compromised as a result of participating in the clinical trial. The HbA1c cut-off of >9.0% was based on the opinions of the trials management team,

consisting of expert endocrinologists, and in accordance with previous clinical trial guidance (Canada Health, 2007).

In addition to study medications, participants continued to receive standard NHS care recommendations concerning life-style modifications (i.e. exercise, weight loss and dietary modification) and management of various co-existing illnesses throughout the trial. Patients were asked to limit alcohol consumption to less than 20 mg/day for females (i.e. 14 units/week) and 30 mg/day for males (i.e. 21 units/week). These levels were consistent with the UK Departmental of Health recommended daily alcohol allowance (British Medical Association, 1995). Participants were not allowed any new prescription or over-the-counter therapies (i.e. herbal remedies, milk thistle) that may improve or worsen NASH throughout the duration of the trial. Potential NASH therapies that were not allowed during the trial duration included TZDs, DPP-4 inhibitors, other GLP-1R agonists (e.g. exenatide), vitamin E and orlistat. Steroids (oral or intravenous), methotrexate and/or amiodarone were also not permitted based on their ability to promote hepatic steatosis.



#### **4.2.4 Outcome Measures**

##### *4.2.4.1 Primary Outcome Measure*

The primary outcome measure is the proportion of participants with a significant improvement in liver histology between liver biopsies at baseline (i.e. within 6 months of screening) and at the end of 48-weeks treatment. The definition of a significant histological improvement requires both the disappearance of steatohepatitis (defined as a disappearance of hepatocyte ballooning) and no worsening of fibrosis, as assessed by the Kleiner scoring system (Kleiner et al., 2005). Hepatocyte ballooning is now widely recognised as the key lesion for distinguishing NASH from simple steatosis.

##### *4.2.4.2 Secondary Outcome Measures*

Secondary outcome measures include changes in; (a) overall NAS (Kleiner et al., 2005); (b) individual histological components of NAS, including lobular inflammation, steatosis, hepatocyte ballooning, and fibrosis; (c) serum markers of steatosis (SteatoTest™), NASH (NashTest™, caspase-cleaved CK-18 M30), and fibrosis (ELF; iQUR Ltd), FibroTest™); (d) Liver stiffness evaluation with Transient Elastography (Fibroscan®, Echosens, Paris, France); (e) Insulin resistance (HOMA-IR); (f) Anthropometric measures including body weight, BMI and waist circumference; (g) Lipid profile and glycaemic control (HbA1c, fasting plasma glucose); (h) serum ALT levels; and (i) health-related quality of life (QoL) (SF-26 version 2.0) and nutrition (Block Brief 2000 Food Frequency Questionnaire questionnaires).

## **4.2.5 Analytical Methods**

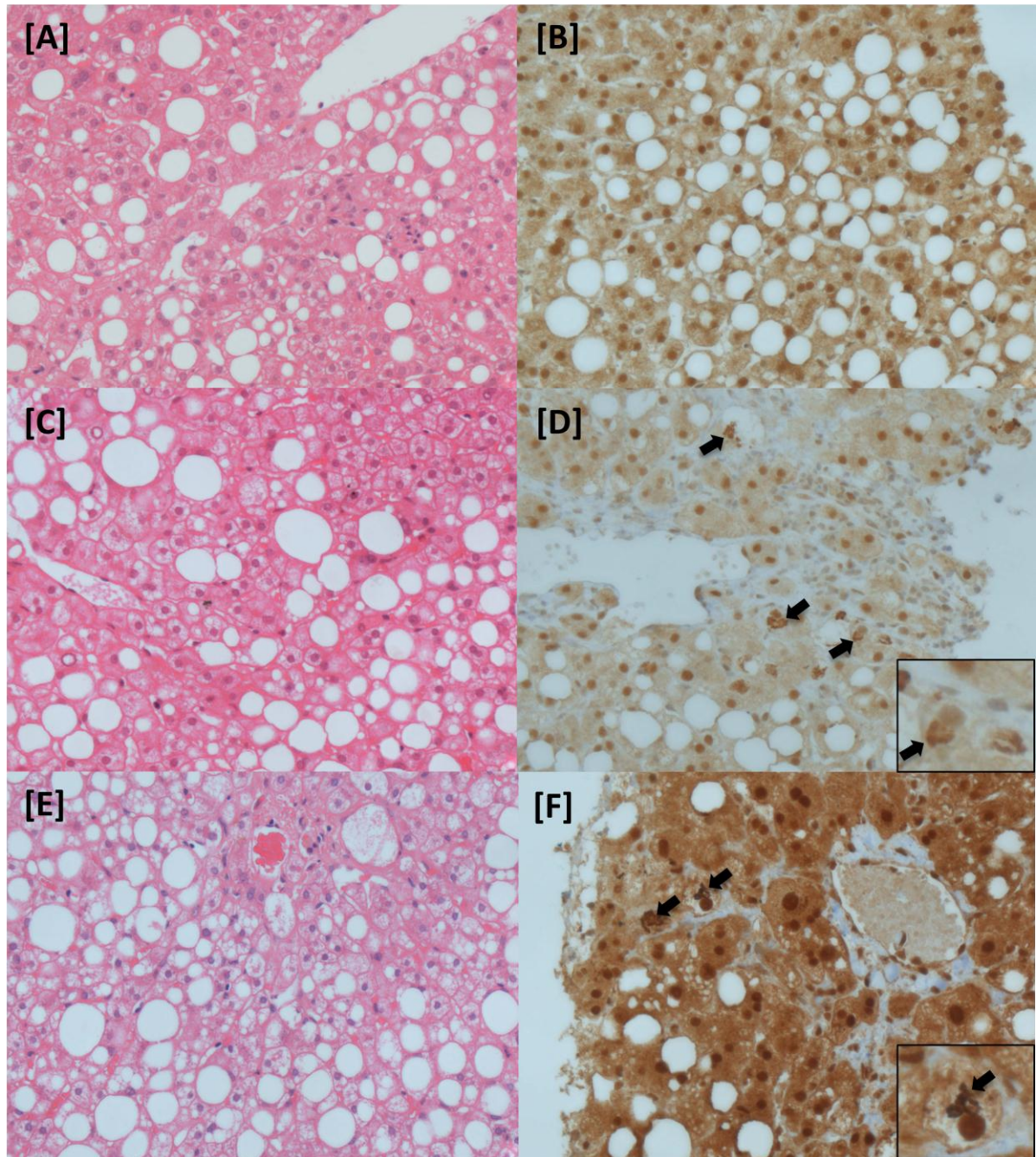
### ***4.2.5.1 Liver Histopathology***

Two independent liver histopathologists (Hübscher, Brown) at the central trial site (Birmingham, UK) will perform all the histopathological assessments using an in-house designed proforma (Table 4-1). Both histopathologists will be blinded to the clinical, laboratory and study treatment allocation. The histological diagnosis of NASH will be established using haematoxylin and eosin (H&E) staining and haematoxylin van Gieson stains of formalin fixed paraffin-embedded liver tissue. Both the baseline and end of treatment (48 weeks) biopsies will be reported as either 'definite NASH,' 'uncertain NASH,' or 'not NASH.' The histological diagnosis of 'definite NASH' is defined as a combination of >5% macrovesicular steatosis, hepatocyte ballooning (+/- Mallory's Hyaline) and lobular inflammation (mixed infiltrate, related to foci of ballooning) (Sanyal et al., 2011). The assessment of ballooning is subjective, and thus for 'uncertain' hepatocyte ballooning, a key component of the diagnosis of NASH, ubiquitin immunohistochemistry will be used to identify material compatible with Mallory's hyaline (Figure 4-2). To validate the quality of the biopsy specimen the core specimen length will be measured and the number of portal tracts will be recorded.

<b>Trial participant</b>	Unique trial ID, date of biopsy, date of review
<b>Diagnosis of NASH on liver biopsy</b>	[ ] definite; [ ] uncertain*; no [ ]
<b>Quality of analysed liver biopsy</b>	Number of complete portal tracts ____ Length of liver specimen (mm) ____
<b>NAFLD Activity Score (NAS) (Kleiner et al., 2005)</b>	<b>Composite score ( _/8)</b>
Steatosis, ( _/3)	0=<5%; 1=5-33%; 2=>33-66%; 3=>66%
Lobular inflammation, ( _/3)	0=No foci; 1=<2 foci/200x; 2=2-4 foci/200x; 3=>4 foci/ 200x
Hepatocyte Ballooning, ( _/2)	0=None; 1=few ballooned cells; 2=many cells/prominent ballooning
<b>Portal tract changes</b>	
Portal inflammation ( _/4) (Ishak et al [34])	0=None; 1=Mild, some or all portal areas; 2=Moderate, some or all portal areas; 3=Moderate/marked, all portal areas; 4=Marked, all portal areas.
Interface hepatitis ( _/4) (Ishak et al,[34])	0=Absent; 1=Mild (focal, few portal areas); 2=Mild/moderate (focal, most portal areas) 3=Moderate (continuous around <50% of tracts or septa); 4=Severe (continuous around >50% of tracts or septa)
Ductular reaction ( _/3)	1= focal in <50% of portal tracts 2= focal in >50% of portal tracts <u>or</u> prominent in <50% of portal tracts. 3=prominent in >50% of portal tracts.
<b>Kleiner Fibrosis Score (F0-F4) (Kleiner et al., 2005)</b>	<i>(select one from the list)</i>
F0	No fibrosis
F1 [1A-1C]	Perisinusoidal OR Periportal [1A=mild, zone 3, perisinusoidal; 1B=moderate, zone 3, perisinusoidal; 1C=portal/periportal]
F2	Perisinusoidal and Portal/periportal
F3	Bridging fibrosis
F4	Cirrhosis
<b>Modified version of Ishak score for fibrosis (Ishak et al., 1995)</b>	<i>(Select one from the list)</i>
0	No fibrosis
1	Zonal fibrosis involving a minority of zone 3 areas and/or portal tracts [specify whether pericellular and/or periportal]
2	Zonal fibrosis involving a majority of zone 3 areas and/or portal tracts [specify whether pericellular and/or periportal]
3	Bridging fibrosis-occasional foci [specify where central-central or central-portal or portal-portal]
4	Bridging fibrosis-widespread [specify where central-central or central-portal or portal-portal]
5	Bridging fibrosis-widespread, with occasional nodule (incomplete cirrhosis)
6	Cirrhosis – probable

**Table 4-1. Trial proforma for the histopathological assessment of pre- and post-treatment liver biopsies.**

Two independent liver histopathologists will perform the histological assessments on the pre and post treatment liver biopsies. \*In the event that one histopathologist reports the diagnosis of NASH as ‘uncertain,’ then a joint review will take place to determine if the participant is eligible for randomization. If both histopathologists regard the case as “uncertain”, this is classed as “no” for eligibility purposes.



**Figure 4-2. Histological inclusion criteria for LEAN trial.**

Liver biopsy sections (actual magnification 400X). **[A – B]** 'Uncertain' NASH - not eligible for LEAN: **[A]** H&E stain highlights fat, inflammation and some pale cells, however **[B]** ubiquitin immunohistochemistry does not identify any Mallory Denk bodies (no confirmed ballooning). **[C – D]** 'Uncertain' NASH - eligible for LEAN: **[C]** H&E stain highlights fat, inflammation and pale cells, but with no obvious Mallory Denk bodies. However, ubiquitin staining **[D]** is positive (confirming ballooned hepatocytes). **[E – F]** 'Definite' NASH - eligible for LEAN: Both H&E and ubiquitin staining highlight fat, lobular inflammation and widespread ballooned hepatocytes. Black arrows highlight Mallory Denk bodies.

The NAS will be calculated based on the Kleiner classification (Kleiner et al., 2005). The NAS is score out of 8, with 8 representing the highest activity. The NAS is the sum of scores of the three components of the histological scoring system, namely steatosis (0 = < 5%, 1 = 5-33%, 2 = >33-66%, 3 = >66%), lobular inflammation (0 = no foci, 1 = <2 foci/200x, 2 = 2-4 foci/200x, 3 = >4 foci) and hepatocyte ballooning (0= none, 1 = few ballooned cells, 2 = many cells/prominent ballooning). The Kleiner scoring system for NAFLD fibrosis (F0-F4) (Kleiner et al., 2005) and a modified version of the Ishak score (Ishak et al., 1995) (F0-F6) (Table 4-1) will be used to evaluate the stage of fibrosis in each biopsy specimen. The Ishak score was modified from the original scoring system, reported in 1995 (Ishak et al., 1995), in order to include the zone 3 peri-cellular/peri-sinusoidal fibrosis, which is characteristically seen in NASH. Portal tract changes (inflammation, interface hepatitis, ductular reaction), an intrinsic feature of NASH, will also be recorded (Brunt et al., 2009).

The pathologists will assess the biopsies independently and fill in separate forms. Cases where there is disagreement on the classification, as 'NASH' or 'not NASH,' will be reviewed and a consensus opinion given. Also discrepancies of more than 1 point on any of the scoring scales (NAS, Kleiner fibrosis scoring system and modified Ishak score) will be reviewed and an amended consensus view offered. Discrepancies of only 1 point will not be altered.

#### *4.2.5.2 Clinical and Laboratory data*

Fasting blood samples will be analysed for full blood count, urea, creatinine and electrolytes, thyroid stimulating hormone (TSH), lipid profile (total cholesterol, HDL, triglycerides), LFTs,

prothrombin time, International Normalised Ratio (INR), amylase, alpha-feta protein (AFP), c-reactive protein (CRP), HbA1c, calcitonin and plasma glucose using standard laboratory methods (Roche Modular system, Roche Ltd, Lewes, UK). Serum Insulin (Mercodia, Uppsala, Sweden), NEFA (Zen-Bio, Research Triangle Park, NC, USA) and CK-18 M30 (M30 Apoptosense ELISA Kit; PEVIVA AB, Bromma, Sweden) will be measured in-house using commercially available colorimetric ELISAs. Serum caspase-cleaved CK-18 M30 and the ELF panel were performed at study entry to assess hepatic apoptosis and fibrosis, respectively.

The FibroMax<sup>TM</sup> panel (consisting of the SteatoTest<sup>TM</sup>, NashTest<sup>TM</sup>, FibroTest<sup>TM</sup>) will be undertaken by Lab 21 Ltd (Cambridge, UK). The ELF test, which combines three direct serum markers of fibrosis (hyaluronic acid, P3NP and TIMP-1) using an algorithm developed by the European Liver Fibrosis Group (Rosenberg et al., 2004), will be performed on fasting serum stored at -80 degrees by a commercial laboratory (iQUR Ltd, Royal Free Hospital, London, UK).

Type 2 diabetes was considered present if patients had a recorded diagnosis in their medical records or if the fasting plasma glucose was  $\geq 7.0$  mmol/L and/or if the 2-hour 75g OGTT plasma glucose was  $\geq 11.1$  mmol/L. All patients without a recorded history of type 2 diabetes were screened with an OGTT. IGT was defined as a 2-hour plasma glucose between 7.8 and 11.1 mmol/L. HOMA-IR was calculated in the standard fashion:  $\text{Glucose (mmol/L)} \times \text{Insulin (uU/L)} \div 22.5$ .

Measurements of weight (kg), height, systolic/diastolic blood pressure and waist:hip circumferences were recorded. Waist and hip circumferences were defined as the circumferential measurements immediately above the level of the iliac crests and at the level of the greater trochanters, respectively. BMI was defined as weight in kilograms divided by the square of the height in metres ( $\text{kg/m}^2$ ).

Liver stiffness was measured using Transient Elastography (Fibroscan®, Echosens, France). The median value and IQR of 10 validated measurements were recorded within the range of 2.5 to 75 kPa. The XL probe was used on individuals who have a BMI greater than  $30 \text{ kg/m}^2$  or when the Fibroscan® 502 Touch machine (automated) recommends its use over the M-probe. To achieve a valid liver stiff evaluation (median of successful liver stiffness measurements) the operator had to obtain all of the following 3 criteria: 1)  $\geq 10$  successful liver stiffness measurements; 2) IQR/median ratio  $< 0.30$ ; and 3)  $\geq 60\%$  measurement success rate (Armstrong et al., 2013b).

#### *4.2.5.3 Patient questionnaires*

QoL was assessed by the Short Form 36 version 2.0 (SF-36v2) health-related QoL questionnaire (QualityMetric Health Outcomes Solutions, Lincoln, USA). The SF-36v2 questionnaire was a practical, reliable and valid measure of physical and mental health that could be completed in 5-10 mins. It consisted of 36 questions that assessed the functional health and well-being from the study subject's point of view (Ware, 2008). The Block Brief 2000 Food Frequency Questionnaire (Block Dietary Data Systems, California, US) was

completed by each subject to assess usual and customary intake of a wide array of nutrients and food groups. The food list incorporated in the Block questionnaire was developed from the National Health and Nutrition Examination Survey (NHANES) III dietary recall data. The Block Brief 2000 Food Frequency Questionnaire is a well-validated self-administered questionnaire, consisting of 70 food related questions and took approximately 15 mins to complete (Block et al., 1990). Pictures of standardized serving sizes and an American-to-English food translation sheet (i.e. 'Catsup' = tomato 'Ketchup') were used to aid completion of the questionnaire.

The Alcohol Use Disorder Identification Test (AUDIT) questionnaire was used to assess the frequency of alcohol consumption and screen out alcohol-related problems, and dependence symptoms (Reinert and Allen, 2002). The AUDIT questionnaire consisted of a 10-item questionnaire that took 2-5 mins to complete. The questionnaire has a positive predictive value of 98% for hazardous drinking, and a negative predictive value of 97% for alcohol dependence. The overall score ranges from 0 to 40, with a score of less than 8 being a good indication of insignificant alcohol consumption.

All questionnaires were completed at baseline (visit 1), end of treatment (visit 7) and 12 weeks post treatment (visit 8). Analysis will report the change from baseline scores to both the end of treatment and follow up scores.



## **4.2.6 Statistical Justification and Outcome Analysis**

### **4.2.6.1 Sample size Justification**

This is an early phase II trial randomising patients equally between two treatment arms - one experimental (liraglutide) and one control (placebo). The primary aim is not to determine the efficacy of liraglutide compared to placebo but to assess whether the efficacy and safety profile of liraglutide is worthy of further investigation. Recruiting patients into a no treatment control group provides simultaneous unbiased assessment of comparable patient groups.

At the time of the study design there were no available data to estimate histological response with 48 weeks treatment of liraglutide (Victoza®). Based on previous non-GLP-1 pharmaceutical trials in NASH utilising improvements in liver histology as a primary endpoint (Lindor et al., 2004; Ratziu et al., 2008), it was assumed that 14-17% of patients undergoing current standard of care (placebo) would have an improvement in NASH by week 48. It was estimated that 20% of the placebo-control arm would achieve an improvement in liver histology, based in part on the knowledge that the placebo-effect might be exaggerated by the subcutaneous injection route of administration (vs. oral route in previous NASH trials) in the current trial. To justify further investigation of liraglutide treatment, a clinically relevant improvement in liver histology was required in at least 50% of patients. The sample size was calculated using A'Hern's single stage phase II methodology (A'Hern, 2001), with a significance level of 0.05 (type 1 error) and power of 0.90 (type II

error). The design required 21 evaluable patients in the treatment group. The published literature in NASH trials reported on average a participant withdrawal rate of 10-20% (Lindor et al., 2004; Aithal et al., 2008; Ratziu et al., 2008). Therefore, to account for a 20% withdrawal rate the recruitment target was inflated from 21 to 25 patients per treatment group; the total recruitment target being 50 patients randomised in a 1:1 allocation ratio to either Liraglutide or placebo.

#### *4.2.6.2 Analysis of Outcome Measures*

All evaluable patients will be analysed on the intention-to-treat principle. Evaluable patients are defined as those who have had an end of treatment biopsy (visit 7), irrespective of the amount of treatment they have received. Patients will be categorised as either achieving the primary histological end-point (i.e. disappearance in NASH) or not. The proportion of patients with a reported improvement in liver histology will be presented and compared across treatments descriptively with 95% confidence intervals. The proposed A'Herns design stipulates that 8 or more evaluable patients out of 21 in the experimental treatment group (liraglutide) need to achieve the defined improvement in liver histology for treatment with liraglutide to be deemed worthy of further investigation with a phase III trial. Analyses will be presented for the subgroups of patients with and without type 2 diabetes. Patients who have not had an end of treatment biopsy will be classed as non-evaluable and will not be included in the primary analysis.

Secondary analysis of the primary outcome measure will report (a) the numbers and proportion of patients that did not have an end of treatment biopsy and the reasons for this (these will be classified as 'no histological improvement') and (b) the numbers and proportion of patients that were considered to have had sufficient treatment and an end of treatment biopsy. In addition, an analysis that directly compares the two proportions for the separate treatment arms will be performed using the Chi-squared test. Secondary outcome measures collected as continuous and categorical variables will be presented with 95% confidence intervals descriptively across treatments using medians and proportions, respectively. Secondary measures collected as longitudinal data (including quality of life data, scored as per the questionnaire specific scoring manuals) will be presented descriptively across treatment groups as changes over time. A summary of all adverse events experienced by patients in both arms will be reported.

## **4.3 Conduct of the trial**

### **4.3.1 Patient Selection**

Eligible adults ( $\geq 18$  years old) were identified and recruited at the participating trial site centres starting in August 2010 and by May 2013, 52 patients were recruited. Participating UK trial centres included the liver units at the Queen Elizabeth UHB (Birmingham, from Aug 2010), Queens Medical Centre (Nottingham, from May 2011), Southampton General Hospital (Southampton, Sept 2011), Hull Royal Infirmary (Hull, Nov 2011) and St. James Hospital (Leeds, from May 2012). All trial participants gave informed written consent at the beginning of the screening visit prior to undergoing any tests and procedures needed to assess eligibility.

Eligibility for the trial was determined at screening visit 1 by standard blood tests, clinical history (including written-confirmation of drug history where necessary) and physical examination/observations to identify other illnesses or contraindications for participation (Trial schedule figure). In addition, after receiving formal training the patient's ability to understand and self-administer the subcutaneous injections using the pre-filled treatment pen was assessed by an experience nurse specialist at screening visit 2. Patients who satisfied the eligibility criteria for the 48 week treatment trial at the Birmingham site were given the option to participate in a metabolic mechanistic sub-study. The sub-study involved two overnight admissions (approximately 22 hours) to the Wellcome Trust Clinical Research Facility (Birmingham) to undergo a 2-step hyperinsulinaemic euglycaemic clamp with stable

isotopes and adipose microdialysis on visits 2 (pre-treatment) and visit 4 (12-weeks treatment). A detailed summary of the metabolic sub-study will be published separately. A patient's decision to partake or withdraw from the metabolic sub-study did not affect their participation in the main 48 week trial.

#### *4.3.1.1 Inclusion Criteria*

The trial entry criteria were based on a diagnosis of 'definite' NASH on liver biopsy obtained within 6 months of screening. Prior to randomisation, two independent liver histopathologists (Hubscher/Brown) from the central trial site (University Hospital Birmingham, UK) reviewed all of the liver biopsies (internal and external trial sites) of the potential participants to assess whether a diagnosis of 'definite' NASH was present. A 'definite' diagnosis of NASH was defined if all of the following were present on biopsy: (i) macrovesicular steatosis (>5%); (ii) hepatocyte ballooning (+/- Mallory Hyaline); and (iii) lobular inflammation (mixed infiltrate, related to foci of ballooning). The two independent histopathology case report forms (CRFs) were reviewed by a trial investigator (Armstrong) and in the event that the histopathologists disagreed with regards to the diagnosis of NASH (i.e. one judged 'uncertain' and the other 'definite') a combined histopathology assessment was undertaken to determine the patient's eligibility status. Only patients with 'definite' NASH (either on two independent reports or after joint review) were classified as eligible.

All participants had to be  $\geq 18$  to  $< 70$  years old and have a body mass index (BMI)  $\geq 25 \text{ kg/m}^2$  at screening. Patients with type 2 diabetes at screening had to have stable glycaemic control

(HbA1c <9.0%) and be managed by either diet and/or a stable dose of metformin/sulphonylurea. Patients without a history of type 2 diabetes prior to the screening visit underwent an OGTT at screening to determine their glycaemic status and were labelled as 'non-diabetic' if one or more of the following was confirmed:

- ❖ IFG, defined using the European Criteria between 6.1 and 6.9 mmol/L
- ❖ IGT, defined as two-hour plasma glucose levels between 7.8 and 11.0 mmol/L on the 75-g OGTT
- ❖ Normal Fasting Plasma Glucose < 6.1 mmol/L and Normal 2-hour plasma glucose levels < 7.8 on the 75g OGTT.

#### *4.3.1.2 Exclusion Criteria*

A detailed summary of the exclusion criteria is provided in Table 4-2. In brief, patients with a history or current significant alcohol consumption, poor glycaemic control (HbA1c > 9.0%), Child's Pugh B or C cirrhosis or another liver disease aetiology were excluded. The latter was confirmed with a full liver aetiology screen (drug-induced, HBV/HCV, autoimmune, and genetic) at the screening visit. Past and current alcohol consumption was ascertained by a detailed review of the patients past medical, social history and by a self-administered AUDIT questionnaire with reference pictures to remind subjects of drink equivalents. Concomitant use of drugs reported to be inducers (methotrexate, amiodarone, steroids) or potential therapies for NASH (TZDs, vitamin E), or other known hepatotoxins were assessed during the screening visit (Table 4-2).

**Generic Exclusion:**

1. Refusal or lacks capacity to give informed consent to participate in the trial
2. Participation in any clinical trial of an investigational therapy or agent within 3 months of randomisation
3. Patient (or carer) deemed not competent at using the correct site and technique for subcutaneous injection of the trial treatment (containing dummy drug on practice)
4. NAFLD Activity Score (NAS) < 3 on liver biopsy
5. Child's B or C cirrhosis
6. Past medical history of multiple drug allergies (defined as anaphylactoid drug reactions in >2 drug groups)
7. Presence of any acute/chronic infections or illness that at the discretion of the chief investigator might compromise the patient's health and safety in the trial
8. Pregnancy or breastfeeding
9. Women, of child-bearing age, who are not willing to practise effective contraception (i.e. barrier, oral contraceptive pill, impenon or past medical history of hysterectomy) for the 48 week duration of the trial and for one-month after the last administration of the drug.
10. Men, sexually active with women of child-bearing age, who are not willing to practise effective contraception for the 48 week duration of the trial and for one-month after the last administration of the drug.
11. Liver disease of other aetiologies (i.e. drug-induced, viral hepatitis, autoimmune hepatitis, PBC, PSC, haemochromatosis, A1AT deficiency, Wilsons disease)
12. Past medical/surgery history of; Gastric bypass surgery, orthotopic liver transplant (OLT) or listed for OLT, hepatocellular, pancreatic, thyroid carcinoma, multiple endocrine neoplasia syndrome type 2 (MEN 2), acute or chronic pancreatitis, and total parenteral nutrition within 6 months of randomisation.
13. Diagnosis of malignancy within the last 3 years (with the exception of treated skin malignancies)
14. Hepatocellular Carcinoma: dysplastic or intermediate nodules to be excluded. Borderline cases to be discussed at Birmingham's tertiary hepato-biliary multidisciplinary team (MDT) meeting. Regenerative and other nodules to be included at the discretion of the chief investigator and the MDT.
15. Family history of medullary thyroid carcinoma
16. Clinical evidence of decompensated chronic liver disease: radiological or clinical evidence of ascites, current or previous hepatic encephalopathy and evidence of portal hypertensive haemorrhage or varices on endoscopy
17. Abnormal clinical examination of thyroid (i.e. unexplained goitre or palpable nodules)
18. ALT or AST > 10 x upper limit of normal
19. Average alcohol consumption/week male >21 (approx. 210g), female >14 units (approx. 140g) within the last 5 years.
20. >5% weight loss since the diagnostic liver biopsy was obtained.
21. Recent (within 3 months of the diagnostic liver biopsy or screening visit) or significant change (as judged by the chief investigator) in dose of the following drugs: Inducers of hepatic steatosis (steroids (oral/intravenous), methotrexate, amiodarone), orlistat and/or multi-vitamins/vitamin E (containing >200% recommended daily amount; >30mg/day)
22. Known positivity for antibody to Human Immunodeficiency virus (HIV)
23. Serum creatinine >150 µmol/L or currently being treated with renal replacement therapy (i.e. Haemodialysis or Peritoneal Dialysis)

**Specific exclusion criteria for subjects with type 2 diabetes:**

1. Current or previous insulin therapy, with exception of previous short-term insulin treatment in connection with intercurrent illness is allowed (≥ 3 months prior to screening), at the discretion of the chief investigator.
2. Subjects receiving thiazolidinediones (TZDs), dipeptidyl peptidase (DPP) IV inhibitors and other GLP-1 based therapies (i.e. exenatide)
3. HbA1c ≥ 9.0%
4. Recurrent major hypoglycaemia or hypoglycaemic unawareness as judged by the chief investigator

**Table 4-2. LEAN trial Exclusion criteria.** Patients who met any of the criteria (listed above) at the screening visit were excluded from trial participation.

In keeping with previous clinical trials assessing GLP-1 therapies, patients with a history of acute/chronic pancreatitis (of any cause), pancreatic and thyroid carcinoma, and/or a family history of medullary thyroid carcinoma were also excluded.

#### **4.3.2 Randomisation**

Subjects who met all the eligibility criteria and provided written informed consent were randomly assigned on a 1:1 basis to either of the two study treatments (liraglutide vs. placebo) using computer generated randomisation at the Cancer Research UK Clinical Trials Unit (CRCTU). The randomisation was stratified to ensure that there were equal numbers of patients with/without type 2 diabetes in each treatment group and that each trial site had equal numbers of patients on each treatment. Trial subjects were allocated a unique trial identification number to preserve patient confidentiality and enable the study to be double-blinded.

#### **4.3.3 Medication preparation and blinding/unblinding procedures:**

Both liraglutide and placebo-control were packaged and labelled with a unique identification number (in keeping with the European Unions Good Manufacturing Practice for Medicinal Product guidelines) in by the manufacturer (Novo Nordisk Ltd), to the extent that the receiving trial site was blinded to the study drug throughout the duration of the trial. Sealed parcels (containing electronic information) were sent with each drug package for the attention of the unblinded members of the central trial management group (nominated



statistician and database programmer) to ensure a) safe delivery of the correct drug and b) blinding of the treatment allocation from the remainder of the trial management group and the trial patient. An independent unblinding service (24/7) was provided by the Medical toxicology and Information services, Guys hospital (London, UK), throughout the duration of the trial.

Unblinding of treatment only take place if the identity of the allocated study medication was necessary for patient safety and care. If a SAE was deemed unexpected and possibly, probably or definitely related to liraglutide (i.e. suspected unexpected serious adverse reaction = SUSAR), a clinical member of the trial management group was unblinded to the medication to evaluate causality. Subsequently, the event was either labelled as an unrelated SAE (for patients receiving placebo) or a SUSAR (for patients receiving liraglutide). The latter were reported to the MHRA and the NRES, and only if patient safety was jeopardised was the study medication discontinued and the treating clinician/patient informed.

#### **4.3.4 Adverse event (AE) reporting and analysis**

The reporting period for AEs commenced at screening visit 1 and continued until follow-up visit 8. SAEs were reported until day 336 (week 48) of the trial treatment and for 30 days post-EOT. All SAEs and adverse reactions were evaluated by the investigators and recorded. The National Cancer Institute's common terminology criteria for AEs (CTCAE, version 4.02, 2010) was used to grade each AE. The central trial office (CRCTU, Birmingham) kept detailed

records of all AEs reported (nature, onset, duration, severity, outcome) and performed an evaluation with respect to seriousness, causality and expectedness. Interim analysis of safety data was performed and presented to the independent data management committee (DMC) on a 6-monthly basis. The unblinded DMC advised accordingly with regards to the trial conduct and specifically whether extra/new data monitoring was required for the remainder of the trial. The DMC operated in accordance with a trial specific charter based upon the template created by the Damocles Group. Specific attention was given to AEs related to the thyroid (measures of blood calcitonin, TSH and physical examination) and pancreas (blood amylase, symptom recognition for pancreatitis), in light of previous non-human (rodents) and post-marketing human safety data (in patients with diabetes), respectively (Alves et al., 2012; Franks et al., 2012).

#### **4.3.5 Study visit overview**

The LEAN trial involved 8 patient-related visits at their nearest trial site. The study was divided into four stages: (1) screening, enrolment, randomisation and baseline investigations (visits 1 and 2, over a maximum period of 14 days), (2) 336 days of study treatment (visits 3,4,5 and 6, over 48 weeks), (3) Primary end-point assessment including liver biopsy (visit 7, within 1 day of the EOT), and (4) post-treatment follow-up assessment (visit 8, 12 weeks after EOT). If the trials investigating team or the trial participant suspected an adverse event, an unscheduled visit was arranged within 24 hours.

The schedule for the study visits and data collection is summarised in Table 4-3.

	Screening		Treatment (TD, treatment day)					Follow-up
	Visit 1 (Max -14 days to TD1)	Visit 2 (1 day prior to TD1)	Visit 3 (TD 28)	Visit 4 (TD 84)	Visit 5 (TD 168)	Visit 6 (TD 252)	Visit 7 (1 Day + TD 336/ End of Treatment [EOT])	
Informed consent	X							
Clinical assessment [1]	X		X	X	X	X	X	X
Vital Signs [2]	X		X	X	X	X	X	X
ECG/Urine Dipstix	X			X	X	X	X	X
Standard blood tests [3]	X		X	X	X	X	X	X
Screening blood tests [4]	X							
Lipid profile;Serum insulin	X			X	X		X	X
OGTT (non-diabetics only)	X						X	
Non-invasive fibrosis markers [5]	X						X	X
Metabolic sub-studies [6]		X		X				
Questionnaires [7]	X						X	X
<b>Liver biopsy</b>	- [8]						X	
Adverse/ Clinical events [9]			X	X	X	X	X	X
Study medication dispensed		X [10]	X	X	X	X		

**Table 4-3. Data collection schedule.**

**[1]** Clinical assessment: complete history/examination (visit 1), focussed history/examination (visits 2-8). **[2]** Vital signs: HR, BP, weight, Height, waist:hip circumference, body temperature, SaO<sub>2</sub>, RR. **[3]** Standard fasting blood tests: FBC, U+E, LFTs, INR, TFTs, glucose and HbA1c (*except visit 3*). **[4]** Screening blood tests: HBsAg, HCV Ab, AMA/ASA/immunoglobulins, Ferritin/Transferrin saturation, Caeruloplasmin,  $\alpha$ 1AT, AFP. **[5]** FibroMAX panel (FibroTest, SteatoTest, NashTest), ELF tests and transient elastography (Fibroscan; optional depending on availability). **[6]** Optional metabolic sub-study: 2-step hyperinsulinaemic euglycaemic clamp with stable isotope studies and adipose microdialysis. **[7]** Questionnaires: AUDIT, Block Brief 2000 FFQ, HR-QOL (SF-36v2). **[8]** Diagnostic liver biopsy performed as part of standard NHS care  $\leq 6$  months of screening visit 1. Two independent liver histopathologists will review the liver biopsy to assess whether the patients meets the histological inclusion criteria. **[9]** Adverse Events/bloods and Clinical Events will be monitored continuously until completion of follow up and 30 days after. Calcitonin and AFP levels will be measured at visits 1, 5, 7 and 8. **[10]** If the study patient meets the eligibility criteria, he/she will be randomised at visit 2 to receive liraglutide (Victoza®) or placebo. The allocated blinded study treatment will be dispensed at visit.

All subjects were asked to attend each visit fasted from eating or drinking (with exception of water) for a minimum of 8 hours prior to each visit. A follow-up liver biopsy (i.e. primary end-point) was obtained under ultrasound-guidance after completion of 48 weeks study treatment. Wherever possible, a 16-gauge biopsy needle and a specimen length of a minimum of 15 mm were preferred. The liver tissue was prepared at the local trial sites in preparation for histological assessment (under light microscopy) at the central trial site at the Queen Elizabeth UHB. On receipt, the two central 'blinded' central histopathologists recorded the size and quality of the histology slides. A minimum of four unstained slides was available for each liver biopsy to enable repeat staining (H&E, haematoxylin van Gieson, Ubiquitin) to ensure adequate quality for interpretation.

#### **4.3.6 Storage of trial samples**

Liver biopsy tissue specimens were collected, paraffin-fixed and stored at the diagnostic archive of the department of cellular pathology (University Hospital Birmingham). Serum and plasma samples collected at visit 1 (screening), visit 4, visit 5, visit 7 (EOT) and visit 8 (12 weeks post EOT) were stored frozen in 0.5-1.0ml aliquots at -80°C at the Institute of Biomedical Research, University of Birmingham. Where possible, additional blood (buffer coat) were obtained at visits 1 and 7 for future DNA extraction and stored at -80°C. Both specimen storage banks hold a licence from the Human Tissue Authority to store tissue for research purposes.

#### **4.3.7 Treatment compliance**

Treatment compliance was monitored by a review of the used pre-filled treatment pens, participant injection sites, and the participants self-filled 'standardised treatment and clinical events booklet' at each study visit. The latter provided written evidence of dosage, time and date when each patient administers the study drug.

#### **4.3.8 Data handling, quality assurance, record keeping and retention**

Data management was undertaken according to the standard operating procedures of the CRCTU at the University of Birmingham, UK. The CRCTU was fully compliant with the Data Protection Act 1998 and the International Conference on Harmonisation Good Clinical Practice (ICH GCP). The CRCTU was responsible for monitoring the trial and providing annual reports to the MHRA. The trial was registered with the Data Protection Act website at the University of Birmingham. Participant identifiable data were shared only within the clinical team on a need-to-know basis to provide clinical care, and to ensure good and appropriate follow-up. Patient identifiable data were also shared with approved auditors from the NRES, Competent authorities (including MHRA, EMA and FDA), Sponsor (University of Birmingham), NHS R&D departments and the primary care practitioner. All LEAN participants provided specific written-consent at trial entry to enable data to be shared with the above. Otherwise, confidentiality was maintained throughout the trial and thereafter. On completion of the trial, data will be transferred to a secure archiving facility at the University of Birmingham, where data will be held for a minimum of 10 years and then destroyed.

#### **4.3.9 Case Report Forms**

Case report forms included baseline/follow-up medical history and physical examinations to capture co-morbidities and concomitant medications in the trials electronic database. Other case report forms incorporated in the electronic database included: laboratory tests and questionnaire results were recorded for visit 1 (eligibility criteria) through to visit 8; safety monitoring during the treatment follow-up periods; central site histopathology reports of liver biopsy specimens; specialist non-invasive markers of liver disease; adverse event reporting; and study drug dispensing forms for study treatment adherence and accountability.

#### **4.3.10 Sponsorship, Indemnity and Monitoring**

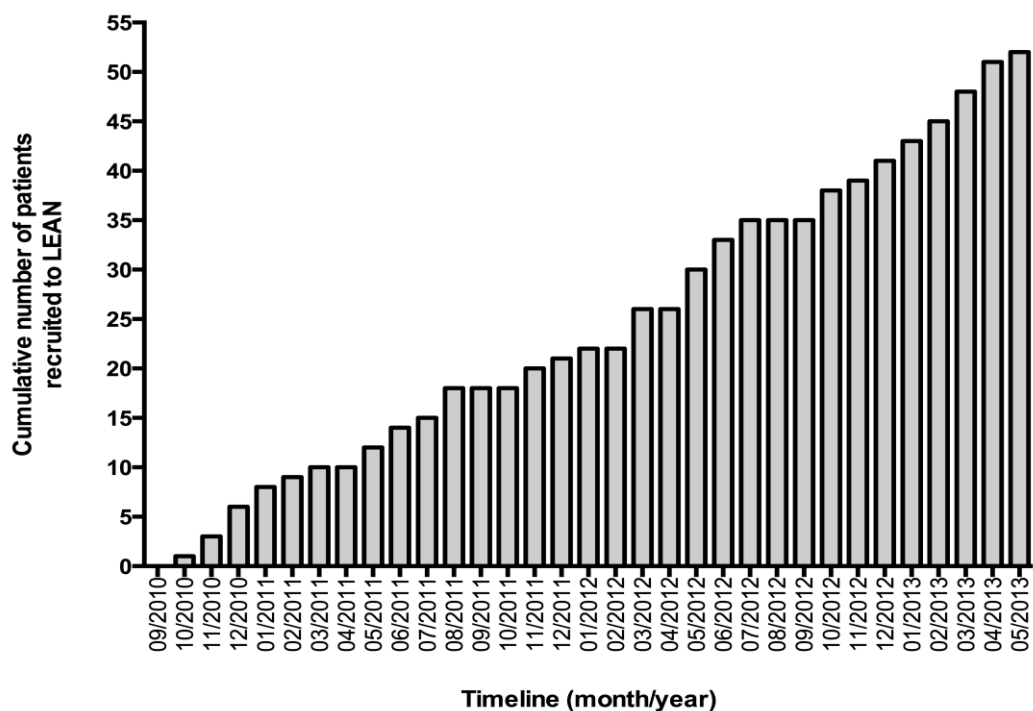
The University of Birmingham acted as the sponsor of the trial. As sponsor the University was responsible for the general conduct of the study and indemnified the trial centre against any claims, arising from any negligent act or omission by the University in fulfilling the sponsor role in respect of the study. Both on-site and off-site monitoring of the trial were performed as per the LEAN Trial Quality Management Plan.

#### **4.3.11 Sources of funding**

The trial was funded by the Wellcome Trust (Clinical Research Fellowship awarded to Armstrong MJ, 2009), Novo Nordisk Ltd (free study drug) and the NIHR liver BRU.

#### 4.4 Trial status

Recruitment into the LEAN trial commenced in August 2010 and ended in May 2013, with 52 patients (104% of target enrolment) randomised from 5 trial sites (Birmingham 31; Nottingham 12; Hull 6; Leeds 3; Southampton 0). This number is 2 more than planned so as to allow all participants that had registered/consented and found to be eligible to participate in the trial. Figure 4.3 summarises the recruitment rate throughout the trial. A total of 73 patients were registered for the trial, 21 (29%) of whom were not eligible or withdrew consent before randomisation to the trial. Failure to meet the histological inclusion criteria (after central histopathology review) was the most frequent reason for ineligibility. The treatment follow-up of LEAN participants is currently ongoing and the last trial visit of the last participant is due to take place in July 2014.



**Figure 4-3. Recruitment rate for LEAN trial between Sept 2010 and May 2013.**

## **4.5 Discussion**

Compliance with the trial protocol and safety profile of liraglutide was reviewed on a bi-annual basis by an independent DMC, and no concerns were raised.

### **4.5.1 Challenges in trial design**

Despite recent advances in non-invasive markers of liver injury (e.g. transient elastography, serum fibrosis markers), liver biopsy remains the recommended method for assessment of disease activity for phase II/III trials (Sanyal et al., 2011). Liver biopsy is not without its limitations (such as sampling error, invasive nature and patient reluctance for repeat sampling (Bravo et al., 2001)), but until the accuracy of serial measurements of non-invasive markers have been formally validated, it will be required for trials in NASH. The LEAN trial has attempted to minimise these limitations. First, liver biopsies (<6 months of screening) performed for routine NHS diagnostic purposes were incorporated into the eligibility criteria and utilised as the baseline comparator, rather than performing two biopsies for the sole purposes of the trial. This approach is widely accepted in trials of NASH. Second, all of the liver biopsies (baseline, primary end-point) underwent a blinded central review by two independent expert liver histopathologists (Hubscher/Brown) at the one site, ensuring that only patients with 'definite' NASH were recruited to the trial and reducing intra/inter-assessor variability, which has previously been reported between trial sites (Sanyal et al., 2010).



In 2011, Sanyal and colleagues (update from AASLD research workshop, 2009) published expert guidance on clinical trial design in patients with NASH (Sanyal et al., 2011). Even though the LEAN trial design preceded this workshop, the definition of NASH and the outcome measures were in keeping with their recommendations. Patients with NASH have a higher risk of liver-related mortality than those with simple hepatic steatosis (+/- mild inflammation) (Ekstedt et al., 2006; Söderberg et al., 2010). Due to the long time-span of NASH progression (i.e. 10-20 years) to end-stage liver failure/death it is impractical to perform therapeutic trials with mortality as the primary outcome measure. Therefore, we elected to use disappearance of NASH with no worsening of fibrosis as 'surrogate' primary end-point in LEAN. With this in mind, 48-weeks treatment duration was selected, rather than 2-5 years, which would be required if we were aiming to demonstrate significant improvements in fibrosis. NAS has been incorporated as a secondary outcome measure (inc. the individual components of NAS) to represent disease activity (Kleiner et al., 2005), rather than as the primary outcome as previously reported (Promrat et al., 2010; Sanyal et al., 2010). NAS alone was not originally designed to infer absence or presence of NASH (Brunt et al., 2011), which we deemed a more meaningful clinical outcome.

We elected to recruit patients with and without type 2 diabetes to enhance recruitment rates and broaden the safety data in liraglutide in NASH, but under the provision that patients with diabetes must have moderate glycaemic control (HbA1c <9.0%) on diet +/- oral hypoglycaemic medications (with the exception of TZDs and other potential confounders i.e. GLP-1 based therapy) prior to trial entry. In the knowledge that diabetes is a potential

confounding factor, randomisation was programmed to stratify for diabetes to ensure equal numbers in each treatment arm.

Efficient recruitment for clinical trials in NASH remains a challenge, mainly due to the requirement for liver biopsy, which has been compounded by the recent uptake of non-invasive markers (e.g. transient elastography) in the UK resulting in a decline in liver biopsy requests in some recruiting centres (Armstrong et al., 2013b).

#### **4.5.2 Safety profile of liraglutide**

Prior to the start of the LEAN trial, the SmPc for liraglutide (Victoza®) stated special warnings and precautions for use in moderate/severe renal impairment, moderate/severe congestive heart failure (NHYA class III/IV), pre-existing thyroid disease and in patients at risk of pancreatitis/pancreatic carcinoma (European Medicines Agency, 2012). In turn, the eligibility criteria (Table 4-2) reflected these warnings by excluding patients with or at risk of such. In particular, based on the pre-clinical incidence of thyroid C-cell tumours in rodent models and the manufacturers 'black box' warning in humans (European Medicines Agency, 2012), all patients with a personal history/family history of thyroid carcinoma, multiple endocrine neoplasia syndrome type 2 and/or abnormal thyroid examination (goiter, nodules) were excluded from the trial. In addition, serum calcitonin, TSH and clinical thyroid examination were monitored throughout the trial as a precautionary measure.

In keeping with both US FDA (US Food and Drug Administration, 2013) and EMA (European Medicines Agency, 2009) recommendations, all patients in LEAN were given written/verbal advice about the risks and carefully monitored for signs and symptoms indicative of pancreatitis. In March 2013, a small study (n=8) by Butler et al reported pancreatic cellular changes, consistent with pancreatic duct metaplasia, in organ donors who had received GLP-1 therapy for diabetes prior to death (Butler et al., 2013a). In response in July 2013, the EMA's committee of Medicinal Products for Human Use (CHMP) critically appraised the study and all other non-clinical/clinical data available, and concluded that the current evidence did not confirm an increased risk of pancreatic adverse events with GLP-1 based therapies (CHMP, 2013). Subsequently, the current safety measures adopted by the LEAN trial will continue until further information is made available.

#### **4.5.3 Summary**

To the best of our knowledge, the LEAN trial is the first multi-centre, double-blinded, placebo-controlled RCT designed to investigate whether the long-acting GLP-1 analogue, liraglutide, is safe and improves liver histology in overweight patients with NASH. The enrolment of the required sample size was completed in May 2013 and the final results are expected by the end of 2014. The full LEAN protocol (version 7.0) can be obtained from the NIHR liver biomedical research unit and CRCTU at the University of Birmingham ([LEAN@trials.bham.ac.uk](mailto:LEAN@trials.bham.ac.uk)).

## **CHAPTER 5: ABDOMINAL SUBCUTANEOUS ADIPOSE TISSUE INSULIN RESISTANCE AND LIPOLYSIS IN NONALCOHOLIC STEATOHEPATITIS**

### **5.1 Introduction**

NASH is associated with a significant risk of developing type 2 diabetes, CKD and CVD morbidity and death (Armstrong et al., 2013a). A better understanding of the key components of the pathogenesis of NASH is therefore needed to provide new therapeutic approaches and thus prevent progressive liver disease and these extra-hepatic complications.

Systemic IR is recognised as one of the main pathogenic factors in NASH (Marchesini et al., 2001; Chitturi et al., 2002). Using hyperinsulinaemic euglycaemic clamp techniques (coupled with stable isotopes), several studies have identified the liver (with increased glucose production) and muscle (decreased glucose disposal) as the key sites of increased IR in patients with NASH (Marchesini et al., 2001; Sanyal et al., 2001; Bugianesi et al., 2005a; Lomonaco et al., 2012; Ortiz-Lopez et al., 2012). Recent studies, however, have recognised the importance of adipose tissue, as the principal source of fatty acids ( $\approx 60\%$ ) for the liver, in driving lipid synthesis in both healthy individuals (Barrows and Parks, 2006) and NASH patients (Donnelly et al., 2005). Adipose tissue is a highly insulin responsive tissue (Cignarelli et al., 2013). In an insulin sensitive state, insulin promotes lipid storage (through fatty acid uptake, re-esterification and hepatic DNL) and inhibits triglyceride lipolysis, the process

whereby triglyceride is hydrolysed to release NEFA from their glycerol backbone. Studies in patients with NASH have inferred changes in adipose tissue insulin sensitivity through systemic measures of circulating NEFA which are elevated in both the fasting state and under hyperinsulinaemic conditions (clamp studies or after oral glucose tolerance testing / meal-challenge) (Sanyal et al., 2001; Bugianesi et al., 2005a; Lomonaco et al., 2012; Musso et al., 2012). Importantly, this appears to be independent of the degree of obesity (Gastaldelli et al., 2009).

Adipose tissue dysfunction is considered to be a major contributory factor of NASH, by means of the resultant 'lipotoxicity' inducing both hepatic IR and skeletal muscle IR (Cusi, 2012). Studies that have been published to date, however, have solely focused on quantifying whole-body lipolysis using either circulating NEFA (i.e. quantified by Adipose IR index = fasting NEFA x insulin) (Lomonaco et al., 2012; Musso et al., 2012; Ortiz-Lopez et al., 2012) or the rate of systemic appearance of labelled glycerol/palmitate isotopes (Sanyal et al., 2001; Fabbrini et al., 2008; Gastaldelli et al., 2009). In particular, no studies have assessed the response of local adipose tissue to the action of insulin, which provides greater insights into the functional relevance of adipose tissue. A greater understanding of which adipose depots are dysfunctional in NASH patients would greatly enhance our knowledge in developing new targeted therapies. Traditionally, visceral adipose tissue (VAT) has been recognised as a major contributor to IR and metabolic conditions including NASH, due to its close proximity to the portal vein and abundance of pro-inflammatory mediators (Fontana et al., 2007; Tordjman et al., 2009). However, as VAT only contributes to 15-20% of circulating NEFA pool (Garg, 2004; Nielsen et al., 2004b), researchers have questioned whether

overspill from abdominal subcutaneous adipose tissue (SAT) plays a more significant role. Indeed, whilst several studies have linked abdominal SAT with indices of insulin resistance in subjects with and without metabolic syndrome (Abate et al., 1995; Abate et al., 1996; Goodpaster et al., 1997; Ferreira et al., 2005), none have examined it in relation to NASH.

Adopting an integrative physiological approach with functional measures of lipid and carbohydrate flux, I have performed a clinical study to determine the relative contribution of tissue-specific insulin sensitivity, notably in SAT, in patients with biopsy-proven NASH in comparison with a healthy control cohort.

## 5.2 Methods

The clinical protocols received full ethical approval from Leicestershire, Northamptonshire & Rutland (ref. 10/H0402/32) and South Birmingham (ref. 10/H1207/15) Local Research Ethics Committees. All adult subjects gave informed written consent prior to participation.

### 5.2.1 Study subjects

#### 5.2.1.1 NASH patients

Sixteen patients with a definitive diagnosis of NASH on liver biopsy within 6 months of the study were recruited. The histological diagnosis was made using well-established criteria (Sanyal et al., 2011) by two independent liver histopathologists. The subjects were of adult age (18-70 years) and had a BMI  $\geq 25$  kg/m<sup>2</sup>. Patients with co-existing type 2 diabetes were diet-controlled or were on a stable dose of metformin +/- glicazide for a minimum of 3 months prior to the study and had a HbA1c <9.0%. Participants were excluded if they had a history of excess alcohol consumption (females >14, males >21 units/week), liver disease of other aetiology, decompensated cirrhosis (Child's Pugh B or C), recent or concomitant drug use of inducers of hepatic steatosis/weight-inducing therapy, and significant medical co-morbidity.

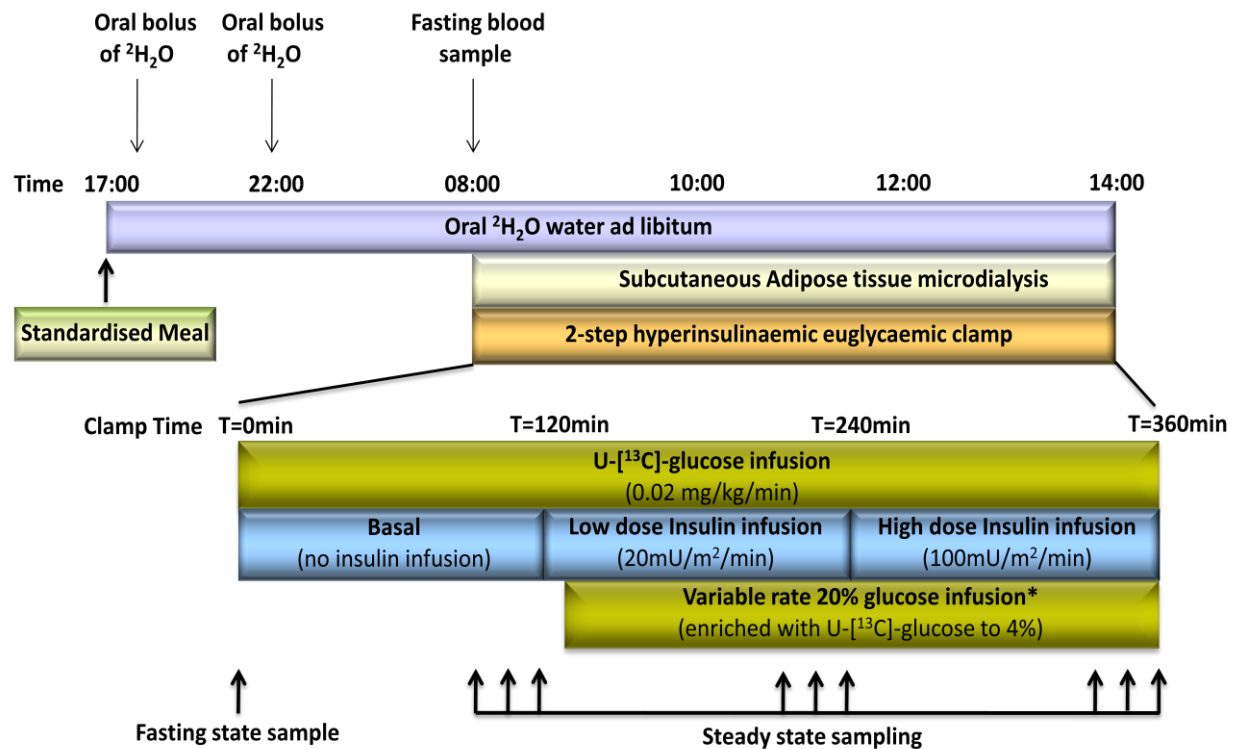
#### *5.2.1.2 Healthy volunteers*

Fifteen healthy volunteers (9 males:6 females; mean age  $33\pm 2$  years) were recruited by use of a local advert. All controls were asymptomatic, non-diabetic, were taking no regular medication and had no significant medical history of note. Female controls had pregnancy excluded and were not taking any form of hormonal contraception. In the healthy control cohort, all consumed alcohol within recommended limits, had normal LFTs and had normal levels of non-invasive markers of hepatic injury (serum CK-18 M30) and fibrosis (ELF panel). Furthermore, all controls had a negative NAFLD Liver Fat Score ( $< -0.640$ ) and estimated liver fat  $< 3.0\%$  based on the Kotronen et al equations, which were originally validated with H-MRS (Kotronen et al., 2009). 5/15 subjects underwent a MRS, as part of a separate study, and in keeping with the Kotronen equations, had hepatic steatosis excluded ( $< 2.5\%$ ).

#### **5.2.2 Study design**

All participants underwent a 2-step hyperinsulinaemic euglycaemic clamp incorporating stable isotopes with concomitant subcutaneous adipose tissue microdialysis at the NIHR/Wellcome Trust Clinical Research Facility (WTCRF, Birmingham, UK) (Figure 5-1).





**Figure 5-1. Schematic of the experiment design**

All participants underwent a 2-step hyperinsulinaemic euglycaemic clamp with stable isotope tracers ( $^{13}\text{C}$ -glucose;  $^2\text{H}_2\text{O}$  deuterated water) and adipose microdialysis to determine tissue-specific insulin resistance. \* variable rate glucose infusion in order to maintain fasting glycaemic control.

#### 5.2.2.1 Hepatic DNL

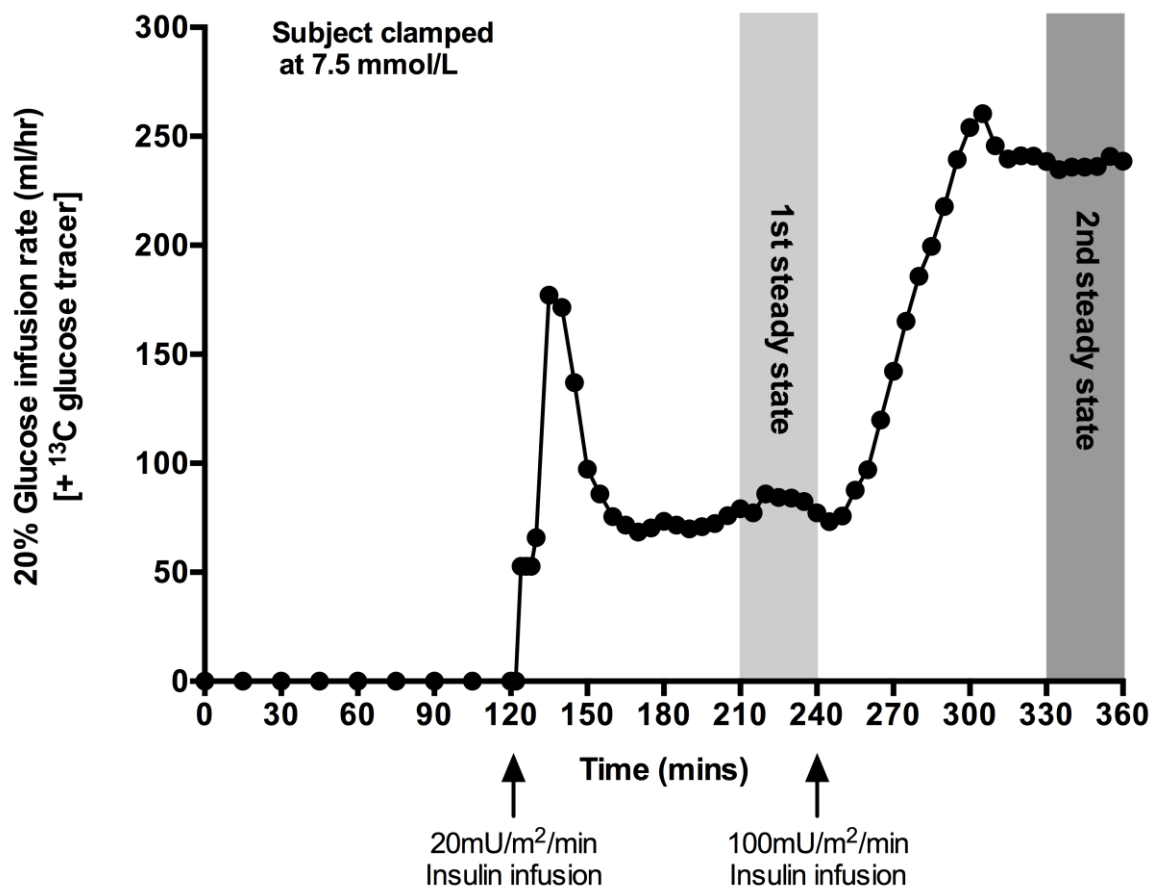
At 17.00 hours, participants were admitted to the WTCRF and total body water was estimated by bioimpedance (Tanita BC418MA, Amsterdam, NL). A standardized meal (carbohydrate 45g, protein 23g, fat 20g) was provided at 17.00 hours, after which participants remained fasted until the end of the clamp at 14.00 hours the next day. To determine rates of DNL, participants were given oral deuterated water,  $^2\text{H}_2\text{O}$  (3g/kg total

body water in 2 divided doses), at 18.00 hours and 22.00 hours followed by ad libitum drinking water enriched with 0.4%  $^2\text{H}_2\text{O}$ .

#### *5.2.2.2 2-step hyperinsulinaemic euglycaemic clamp*

At 08.00 hours the next morning fasting blood samples were taken prior to starting the 2-step hyperinsulinaemic euglycaemic clamp. Arterialised blood was sampled to determine the blood glucose concentration at which to maintain ('clamp') the participant throughout the study using an YSI 2700 machine (YSI life sciences, UK). An intravenous bolus of U- $^{13}\text{C}$ -glucose (2mg/kg body weight; CK gas limited, Hook, UK) was administered over 1 minute followed by a constant infusion rate (0.02mg/kg/min) for 6 hours until the end of the clamp. Steady state blood samples were taken at 3 time points during the final 30 minutes of the 2-hour basal phase. At 10.00 hours, low-dose insulin (Actrapid; Novo Nordisk, Copenhagen, Denmark) was infused at 20mU/m<sup>2</sup>/min. At 10.04 hours a concomitant infusion of 20% glucose enriched with U- $^{13}\text{C}$ -glucose to 4% was commenced. Arterialised blood samples were taken at 5 minutely intervals and the 20% glucose infusion rate changed to maintain fasting glycaemic levels. Steady state blood samples were taken at 3 time points in the final 30 minutes of the 2-hour low-dose insulin infusion (Figure 5-1).

The insulin infusion rate was then increased to 100mU/m<sup>2</sup>/min (high-dose) for 2 hours with sampling as described above. Rates of hepatic endogenous glucose production (EGP) and glucose disposal (Gd) were calculated by using modified versions of the Steele Equations (Steele, 1959; Finegood et al., 1987).



**Figure 5-2. Example of 2-step hyperinsulinaemic clamp from the study**

The infusion rate of 20% glucose (+ 4%  $^{13}\text{C}$ -glucose isotope) was adapted every 5 mins to maintain euglycaemia (7.5 mmol/L in current case) throughout 2 hrs low-dose insulin (20mU/m<sup>2</sup>/min) and 2 hrs high-dose insulin (100mU/m<sup>2</sup>/min). Steady-state samples were collected between 210-240 mins (1<sup>st</sup> step, representing hepatic insulin sensitivity) and 330-360 mins (2<sup>nd</sup> step, representing peripheral insulin sensitivity).

#### 5.2.2.3 Adipose microdialysis

A microdialysis catheter (CMA microdialysis, Solna, Sweden) was inserted after local anaesthetic (5ml 1% lignocaine) into the abdominal SAT (minimum depth 1cm), 10cm lateral to the umbilicus, prior to commencing the clamp. Thereafter, microdialysate samples were collected into microvials (0.3µL/min) every 30 minutes until the end of the clamp.

### 5.2.3 Data Collection and Analysis

#### 5.2.3.1 *Clinical and biochemical parameters:*

Participant demographics and clinical/biochemical measures were recorded at the study visit. Type 2 diabetes was defined by past medical history, 75g 2-hour oral glucose tolerance test, glycosylated haemoglobin HbA1c and/or fasting glucose (WHO 2011). Measurements of weight (kg), height, systolic/diastolic blood pressure and total body/truncal fat mass (bioimpedance) were recorded. BMI was defined as weight in kilograms divided by the square of the height in metres ( $\text{kg/m}^2$ ). Fasting blood samples (0800 hours) were analysed for full blood count, urea, creatinine and electrolytes, TSH, lipid profile, LFTs, HbA1c and plasma glucose using standard laboratory methods (Roche Modular system, Roche Ltd, Lewes, UK).

Serum Insulin was measured using a commercially available colorimetric ELISA (Mercodia, Uppsala, Sweden), with an in-house coefficient of variation of <5%. Serum NEFA were measured in-house using a colorimetric commercial assay (Zen-Bio, Research Triangle Park, NC, USA), with a coefficient of variation between 8.0-9.1%. Both were performed according to the manufactures' instructions.

Serum CK-18 M30 and ELF panel were performed at study entry to assess hepatic apoptosis and fibrosis, respectively. Serum CK-18 M30 was measured in accordance with the manufacturers' guidance using a commercially available colorimetric ELISA (M30

Apoptosense ELISA Kit; PEVIVA AB, Bromma, Sweden), with an in-house coefficient of variation of <5%. The ELF test, which combines three direct serum markers of fibrosis (hyaluronic acid, P3NP and TIMP-1) using an algorithm developed by the European Liver Fibrosis Group (Rosenberg et al., 2004), was performed on fasting serum stored at -80 degrees by a commercial laboratory (iQUR Ltd, Royal Free Hospital, London, UK).

#### *5.2.3.2 Circulating adipocytokines and inflammatory markers*

Fasting serum levels of Adiponectin, Leptin, Resistin, TNF- $\alpha$ , hs-CRP, IL-6, IL-17, CCL-2 (aka MCP-1), CCL-3 (aka Macrophage Inflammatory Protein 1 $\alpha$ ), CCL-4 (aka Macrophage Inflammatory Protein 1 $\beta$ ) and CCL-5 (aka Regulated on Activation, Normal T cell Expressed and Secreted [RANTES]) were quantified using the commercially available multiplex bead immunoassays (Fluorokine® Multi-Analyte Profiling; R&D Systems, Abingdon, United Kingdom) for the Luminex™ 200 Platform (Luminex Corporation, The Netherlands). Six-point standard curves for each cytokine on the Human Obesity Base Kit (cat. No. LOB000) and Human Base Kit A (cat. No. LOB000) were generated by reconstitution of the Standard Cocktail with Calibrator Diluent RD6-46, as per manufacturers instructions. The Biorad Bioplex-Manager (version 6.0) software was used on the Luminex™ 200 machine for acquisition and analysis. The in-house minimum detection limits (coefficient of variations, CV %) were as follows: 438.5 pg/ml for Adiponectin (CV 2.1-9.5%); 58.7 pg/ml for Leptin (CV 2.0-8.1%); 46.4 pg/ml for Resistin (CV 0.9-5.2%); 1.19 pg/ml for TNF- $\alpha$  (CV 1.1-6.6%); 36.0 pg/ml for hs-CRP (CV 0.0-9.0%); 0.46 pg/ml for IL-6 (CV 1.8-6.7%); 0.45 pg/ml for IL-17 (CV 0.44-

20.9%); 3.75 pg/ml for CCL-2 (CV 0.59-9.4%); 18.5 pg/ml for CCL-3 (CV 0.0-12.8%); 8.10 pg/ml for CCL-4 (CV 0.77-13.8%); and 2.96 pg/ml for CCL-5 (CV 0.71-18.7%).

#### *5.2.3.3 Stable Isotope Mass Spectrometry analysis*

The enrichment of U- $^{13}\text{C}$ -glucose in plasma was determined by gas chromatography-mass spectrometry (model 5973; Agilent technologies, Cheshire, UK). Deuterium enrichment of the body water pool was measured using the Gasbench II (Thermo Scientific Inc., Bremen, Germany) ([www.thermo.com](http://www.thermo.com)), an automated  $\text{H}_2/\text{H}_2\text{O}$  equilibration device, coupled on line to a ThermoFinnigan Deltaplus XP Isotope Ratio Mass Spectrometer (IRMS; ThermoFinnigan MAT GmbH, Bremen, Germany). The full methods have been previously described in detail (Hazlehurst et al., 2013). In brief, after adding 200 $\mu\text{l}$  of plasma sample and inserting platinum catalyst to a borosilicate sample vial, the vial is capped and automatically flushed with 2%  $\text{H}_2$  in He equilibration gas. After an equilibration time of 40 minutes, the  $^2\text{H}/^1\text{H}$  enrichment of the head space gas is sampled and analysed automatically (mean of 10-fold measurement) on the IRMS using 2%  $\text{H}_2$  in He as reference gas. The in house coefficient of variation of this assay is <2% for naturally enriched samples and <0.5% for samples with a  $^2\text{H}/^1\text{H}$  ratio 0.001 > natural background.

Deuterium enrichment in the palmitate fraction of total plasma triglycerides was measured on an automated GC/TC/IRMS system (Thermo Finnigan Delta Pus XP; Thermo Electron Cooperation, Bremen, Germany) ([www.thermo.com](http://www.thermo.com)). In brief, the lipid fraction was extracted from 1.5 ml of plasma as described by Folch et al (Folch et al., 1957) and the

triglyceride fraction isolated by solid phase extraction (Bond Elut NH<sub>2</sub>-Aminopropyl columns). After transmethylation of the triglyceride fraction (Lepage and Roy, 1986), the <sup>2</sup>H/<sup>1</sup>H ratio in palmitate methylester was measured via a GC separation of the methylated fatty acids followed by pyrolytic conversion of the palmitate methylester into CO and H<sub>2</sub>, followed by online continuous flow measurement of the <sup>2</sup>H/<sup>1</sup>H ratio in the separated H<sub>2</sub> peak by the ThermoFinnigan Deltaplus XP IRMS (Bremen, Germany). The in house coefficient of variation of this assay is <5% over the sample range observed in this study (<sup>2</sup>H/<sup>1</sup>H ratio 0.00000-0.0004 > natural background). The <sup>2</sup>H/<sup>1</sup>H ratio of both the body water pool and of the palmitate fraction of total plasma triglyceride were corrected against enrichment curves.

#### *5.2.3.4 Abdominal SAT microdialysis:*

Microdialysate samples were analysed using a mobile photometric, enzyme-kinetic analyzer (CMA Iscus Flex, Sweden) for glycerol concentration. The rate of interstitial glycerol release represented the magnitude of SAT lipolysis in the fasted state and in response to insulin.

#### *5.2.3.5 Contribution of Hepatic DNL to total palmitate synthesis*

The percentage contribution of hepatic DNL to endogenous palmitate synthesis was determined by the incorporation of <sup>2</sup>H<sub>2</sub>O in the palmitate present in the plasma total triglyceride pool, as previously described (Hazlehurst et al., 2013). This percentage was calculated from the increase in the <sup>2</sup>H/<sup>1</sup>H ratio in the palmitate methylester of the total triglyceride fraction and in the water of plasma samples taken before (1700 hours, at

admission) and 14 hours after the initial ingestion of the  $^2\text{H}_2\text{O}$  tracer (0800 hours, before the start of the hyperinsulinaemic euglycaemic clamp). The following formula was used: % hepatic DNL contributes to endogenous palmitate synthesis =  $[\text{delta } ^2\text{H}/^1\text{H} \text{ ratio in palmitate methylester}]/[\text{delta } ^2\text{H}/^1\text{H} \text{ ratio in waterpool}] \times (34/22) \times 100\%$ . In the equation, 34 is the total number of H-atoms in palmitate methylester and 22 is the number of water molecules incorporated into palmitate *via* DNL as observed in previous rodent studies (Diraison et al., 1996) and currently used in human studies (Diraison et al., 1997).

#### **5.2.4 Statistical analysis**

Descriptive statistics were applied to characterise the NASH and healthy volunteer cohorts. Continuous clinical and laboratory variables are reported as means and standard error (SE) as all variables had parametric distribution on D'Agostino and Pearson Omnibus Normality testing. Categorical variables are reported as number and percentages. Area under the curve (AUC) analysis was performed using the trapezoidal method for interstitial glycerol release during the clamp. For comparison of single variables, unpaired Student t-tests were used (or non-parametric equivalents where data were not normally distributed). Where repeated samples were taken repeated-measures one-way ANOVA was used, incorporating the Dunnett's test for multiple comparisons. The significance level was set at  $p < 0.05$ . All analysis was performed using the GraphPad Prism 5.0 software package.



## 5.3 Results

### 5.3.1 Participant characteristics

Participant demographics and clinical characteristics are summarised in Table 5-1. The patients with NASH subjects were significantly older ( $54.4 \pm 2.1$  vs.  $33.1 \pm 2.2$  yrs;  $p < 0.0001$ ) and had a higher BMI ( $34.3 \pm 1.0$  vs.  $26.7 \pm 1.0$  kg/m<sup>2</sup>;  $p < 0.0001$ ) and abdominal fat mass on bioimpedance ( $20.3 \pm 1.5$  vs.  $12.0 \pm 1.5$  kg;  $p = 0.0011$ ). Of the 16 subjects with NASH, 5 had mild-moderate fibrosis (Kleiner F1-F2) and 9 had advanced fibrosis (F3-F4). NASH subjects had significantly higher serum levels of liver enzymes (ALT  $68.7 \pm 11$  vs.  $18.9 \pm 2.6$  IU/L;  $p = 0.0001$ ), serum CK-18 M30 levels ( $544 \pm 116$  vs.  $161 \pm 9.8$  IU/L;  $p = 0.0034$ ) and ELF test ( $9.20 \pm 0.3$  vs.  $7.34 \pm 0.1$ ;  $p < 0.001$ ); values for all these parameters were within accepted reference ranges in the healthy volunteers.

### 5.3.2 Systemic IR

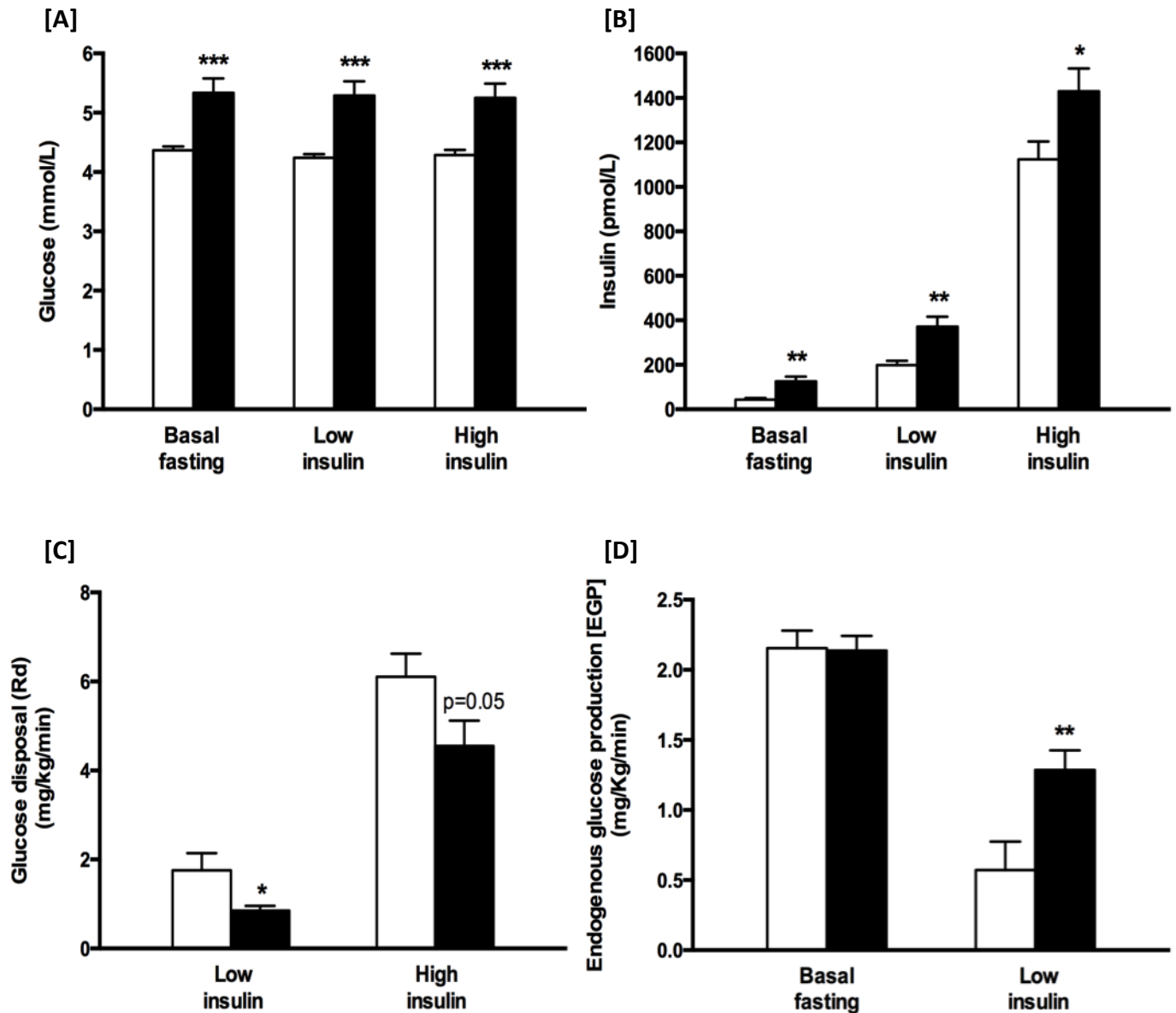
Fasting serum glucose, insulin and HOMA-IR ( $4.40 \pm 0.8$  vs.  $1.19 \pm 0.2$ ) were significantly higher in patients with NASH (all  $p < 0.001$ ) (Figure 5-3A-B). During the 2-step hyperinsulinaemic clamp, NASH subjects had significantly lower weight-adjusted glucose infusion rates in response to low-dose ( $1.47 \pm 0.08$  vs.  $3.08 \pm 0.4$  mg/kg/min;  $p = 0.0008$ ) and high-dose insulin ( $5.80 \pm 0.4$  vs.  $9.14 \pm 0.5$  mg/kg/min;  $p < 0.0001$ ). In keeping with peripheral (largely muscle) IR, weight-adjusted glucose disposal (Gd) rates were significantly lower in NASH subjects at low-

dose ( $0.85 \pm 0.1$  vs.  $1.76 \pm 0.4$  mg/kg/min;  $p < 0.05$ ) and high-dose insulin infusions ( $4.55 \pm 0.6$  vs.  $6.10 \pm 0.5$  mg/kg/min;  $p = 0.05$ ) (Figure 5-3C).

	<b>NASH (n=16)</b>	<b>Controls (n=15)</b>	p-value
<b>Demographics</b>			
Male sex, n (%)	11 (68.8)	9 (60.0)	0.716
Age (years)	54.4 (2.1)	33.1 (2.2)	<b>&lt;0.0001</b>
Caucasian ethnicity, n (%)	16 (100)	14 (93.3)	0.484
Asian ethnicity, n (%)	0 (0)	1 (6.7)	
<b>Metabolic parameters</b>			
Type 2 Diabetes, n (%)	7 (43.8)	0 (0)	<b>0.001</b>
Impaired glucose tolerance, n (%)	3 (18.8)	0 (0)	
Normal glucose tolerance, n (%)	6 (37.5)	15 (100)	
Fasting glucose (mmol/L)	5.34 (0.24)	4.37 (0.067)	<b>0.0008</b>
Fasting insulin (pmol/L)	125.8 (20.8)	43.3 (7.41)	<b>0.0003</b>
Pre-study OAD treatment, n (%)	8 (50.0)	0 (0)	<b>0.0024</b>
Pre-study statin treatment, n (%)	7 (43.8)	0 (0)	<b>0.0068</b>
Pre-study anti-hypertensive treatment, n (%)	6 (37.5)	0 (0)	<b>0.0177</b>
BMI (Kg/m <sup>2</sup> )	34.3 (1.04)	26.7 (0.95)	<b>&lt;0.0001</b>
Weight (Kg)	100.3 (3.83)	78.5 (3.67)	<b>0.0003</b>
Total fat mass (Kg)	35.8 (2.64)	20.2 (1.79)	<b>&lt;0.0001</b>
Truncal fat mass (Kg)	20.3 (1.45)	12.0 (1.51)	<b>0.0011</b>
Systolic BP (mmHg)	129.4 (3.43)	129.3 (2.74)	0.982
Total cholesterol (mmol/L)	4.51 (0.20)	4.59 (0.30)	0.891
HDL (mmol/L)	1.11 (0.064)	1.26 (0.11)	0.256
LDL (mmol/L)	3.01 (0.21)	2.73 (0.43)	0.550
Triglycerides (mmol/L)	1.95 (0.26)	1.62 (0.34)	0.438
TSH (μU/L)	2.76 (0.38)	2.01 (0.31)	0.165
Creatinine (μmol/L)	71.3 (3.46)	72.6 (3.47)	0.800
<b>Liver parameters</b>			
AST (IU/L)	55.1 (5.66)	20.4 (1.55)	<b>&lt;0.0001</b>
ALT (IU/L)	68.7 (10.6)	18.9 (2.57)	<b>0.0001</b>
ALP (IU/L)	77.0 (7.6)	28.3 (4.14)	<b>&lt;0.0001</b>
Bilirubin (μmol/L)	13.4 (1.76)	12.0 (1.02)	0.494
Albumin (g/L)	47.1 (0.70)	41.6 (0.77)	<b>&lt;0.001</b>
Platelets (x10 <sup>9</sup> /L)	203.3 (14.7)	216.1 (10.5)	0.489
CK-18 M30 (IU/L)	543.4 (115.8)	160.9 (9.83)	<b>0.0034</b>
ELF test	9.20 (0.30)	7.34 (0.12)	<b>&lt;0.0001</b>

**Table 5-1. Clinical demographics and characteristics of 16 patients with NASH and 15 ‘healthy’ controls.**

Mean (SE), unless stated. Blood parameters were fasted. Comparisons of continuous variables with unpaired Student’s T test, and categorical variables with fisher chi-squared.



**Figure 5-3. Subjects with NASH have significant systemic, muscle and hepatic IR.**

Circulating glucose **[A]** and insulin **[B]** concentrations during the 2-step hyperinsulinaemic euglycaemic clamp. The degree of muscle and hepatic insulin sensitivity was determined by glucose disposal **[C]** and suppression of hepatic glucose production **[D]**, respectively. Key: White bar = controls, black bar = NASH. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. controls.

### 5.3.3 Hepatic IR

Although fasting EGP rates were similar in patients with NASH and healthy controls ( $2.14 \pm 0.1$  vs.  $2.15 \pm 0.1$  mg/kg/min;  $p > 0.9$ ) (Figure 5-3D), this was in the context of fasting hyperinsulinaemia (Figure 5-3B, Table 5-1), which is consistent with hepatic IR. The hepatic IR index (= EGP x fasting insulin (Gastaldelli et al., 2007)) was significantly higher in the NASH patients ( $278 \pm 52.7$  vs.  $90.0 \pm 14.9$  mg/kg/min.pmol/ml;  $p = 0.0024$ ). Endorsing this observation, low -dose insulin-mediated suppression of EGP was decreased in patients with NASH (Figure 5-3D), consistent with hepatic IR ( $41.0 \pm 4.3$  vs.  $70.2 \pm 9.5\%$ ;  $p = 0.008$ ). These differences persisted even after removing patients with type 2 diabetes ( $n = 7$ ) from the NASH cohort ( $42.2 \pm 5.6$  vs.  $70.2 \pm 9.5\%$ ;  $p < 0.05$ ).

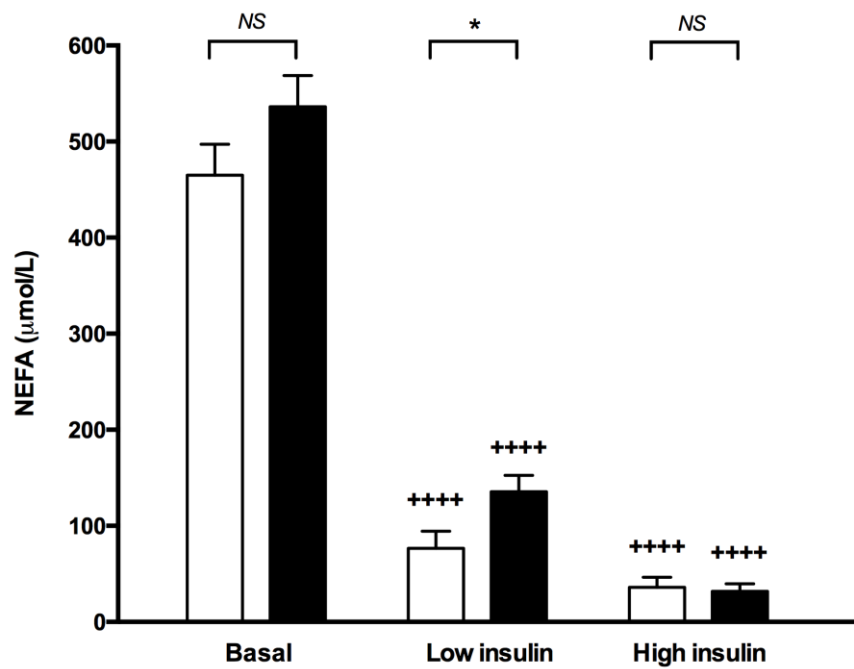
### 5.3.4 Hepatic DNL

The percentage contribution of DNL to total endogenous palmitate synthesis was variable across all individuals and although higher in NASH subjects compared to controls (median  $4.90$  [IQR  $3.9$ - $5.6$ ] vs.  $2.79$  [ $1.2$ - $6.4$ ];  $p = 0.16$ ) this did not reach significance.

### 5.3.5 Depot-specific adipose tissue IR

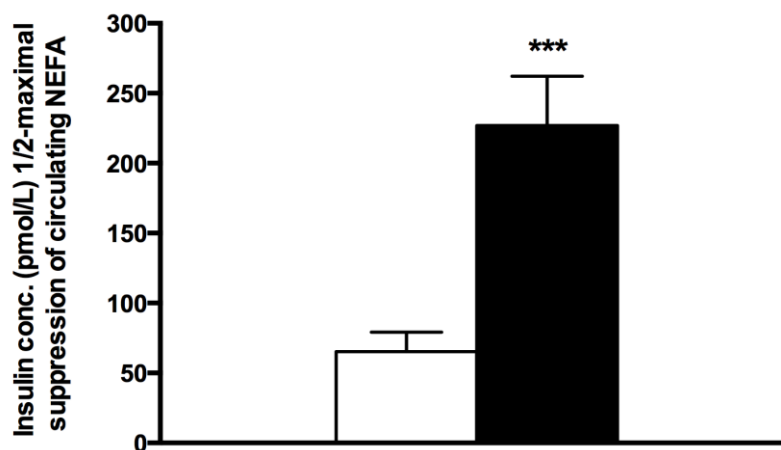
Circulating fasting NEFA levels were not different between patients with NASH and healthy controls ( $563 \pm 33$  vs.  $465 \pm 32$   $\mu$ mol/L;  $p = 0.13$ ) (Figure 5-4). However, taking into account fasting hyperinsulinaemia in patients with NASH, the calculated adipose IR index (fasting

NEFA x fasting insulin (Gastaldelli et al., 2007)) was significantly elevated ( $64.4 \pm 9.1$  vs.  $20.5 \pm 3.9$  mmol/L.pmol/L;  $p=0.0002$ ) in patients with NASH. Insulin infusion significantly suppressed circulating NEFAs in both NASH and control subjects ( $p<0.0001$  vs. basal NEFA in each group) (Figure 5-4). In order to determine insulin sensitivity, using regression analysis, the insulin concentrations causing half-maximal suppression of serum NEFA (INS- $\frac{1}{2}$ -max NEFA) were calculated for each subject (Figure 5-5). INS- $\frac{1}{2}$ -max NEFA was >3-fold higher in NASH subjects compared to the controls ( $227 \pm 35$  vs.  $65.2 \pm 14$  pmol/L;  $p=0.0003$ ) consistent with adipose tissue IR. The significant difference in INS- $\frac{1}{2}$ -max NEFA remained ( $195 \pm 30$  vs.  $65.2$  pmol/L;  $p=0.0002$ ) after removing patients with type 2 diabetes ( $n=7$ ) from the analysis.



**Figure 5-4. Subjects with NASH have significant global adipose IR**

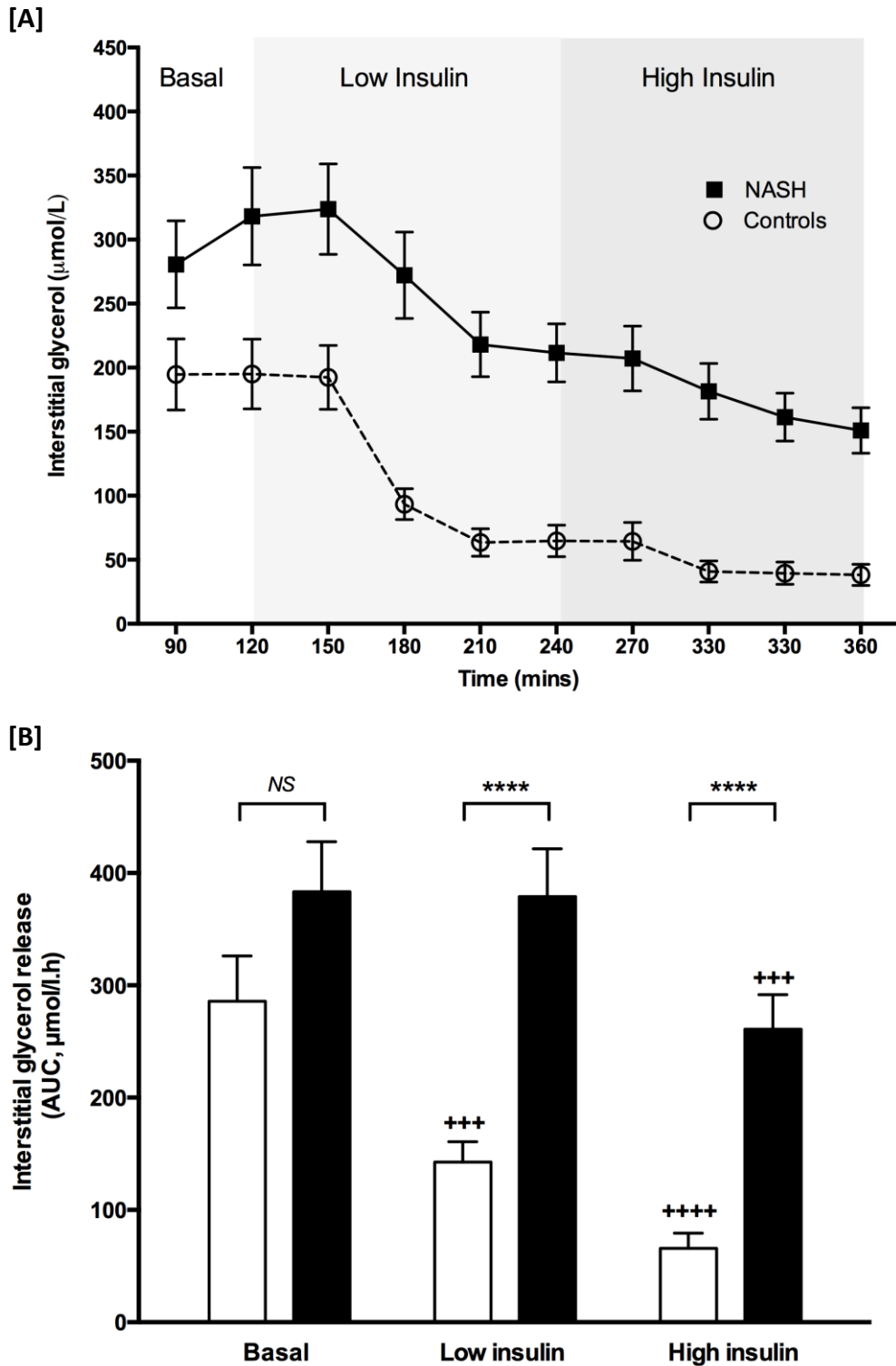
Circulating NEFA concentrations at basal and hyperinsulinaemic phases of euglycaemic clamp. Key: White bar = controls, black bar = NASH. \* $p<0.05$  vs. controls. ++++  $p<0.0001$  vs. basal phase. NS = non-significant.



**Figure 5-5. Subjects with NASH have significant global adipose IR**

As a marker of global adipose tissue insulin resistance, the concentration of circulating insulin concentrations (pmol/L) causing 1/2-maximal suppression of circulating NEFA was calculated. Key: White bar = controls, black bar = NASH. \*\*\*p<0.001 vs. controls.

Interstitial glycerol release assessed using microdialysis, was used as a direct measure of abdominal SAT function (Figure 5-6A). In the fasting state, the rate of interstitial glycerol release was not different in NASH subjects compared to controls ( $383 \pm 44$  vs.  $286 \pm 40$   $\mu\text{mol/L.hr}$ ;  $p=0.12$ ). In healthy controls, low-dose insulin infusion ( $20\text{mU/m}^2/\text{min}$ ) significantly suppressed the rate of interstitial glycerol release (Basal:  $286 \pm 40$  vs. low-dose insulin:  $143 \pm 18 \mu\text{mol/L.hr}$ ;  $p<0.001$ ), whereas it did not suppress the rate of interstitial glycerol release in NASH subjects (Basal:  $383 \pm 44$  vs. low-dose insulin:  $379 \pm 43 \mu\text{mol/L.hr}$ ;  $p>0.05$ ). High dose insulin ( $100\text{mU/m}^2/\text{min}$ ) suppressed glycerol release in both patients with NASH and controls, however, the rate of glycerol release remained significantly higher in the NASH subjects compared to controls ( $261 \pm 31$  vs.  $65.8 \pm 14 \mu\text{mol/L.hr}$ ;  $p<0.0001$ ) (Figure 5-6B). Furthermore, the  $\text{INS-}\frac{1}{2}\text{-max}$  glycerol was 6-fold higher in the NASH subjects compared to controls ( $p<0.0001$ ) (Figure 5-7, bottom right panel).



**Figure 5-6. NASH is associated with significant abdominal SAT IR.**

**[A]** SAT interstitial fluid concentrations of glycerol during the 2-step hyperinsulinaemic euglycaemic clamp. **[B]** To determine the rate of lipolysis in SAT under basal and hyperinsulinaemic conditions area under the curve (AUC) analysis was performed for interstitial glycerol release. Broken line/White bar = controls, solid line/black bar = NASH. \*\*\*\*p<0.0001 vs. controls; +++p<0.001, ++++p<0.0001 vs. basal phase. NS = non significant.

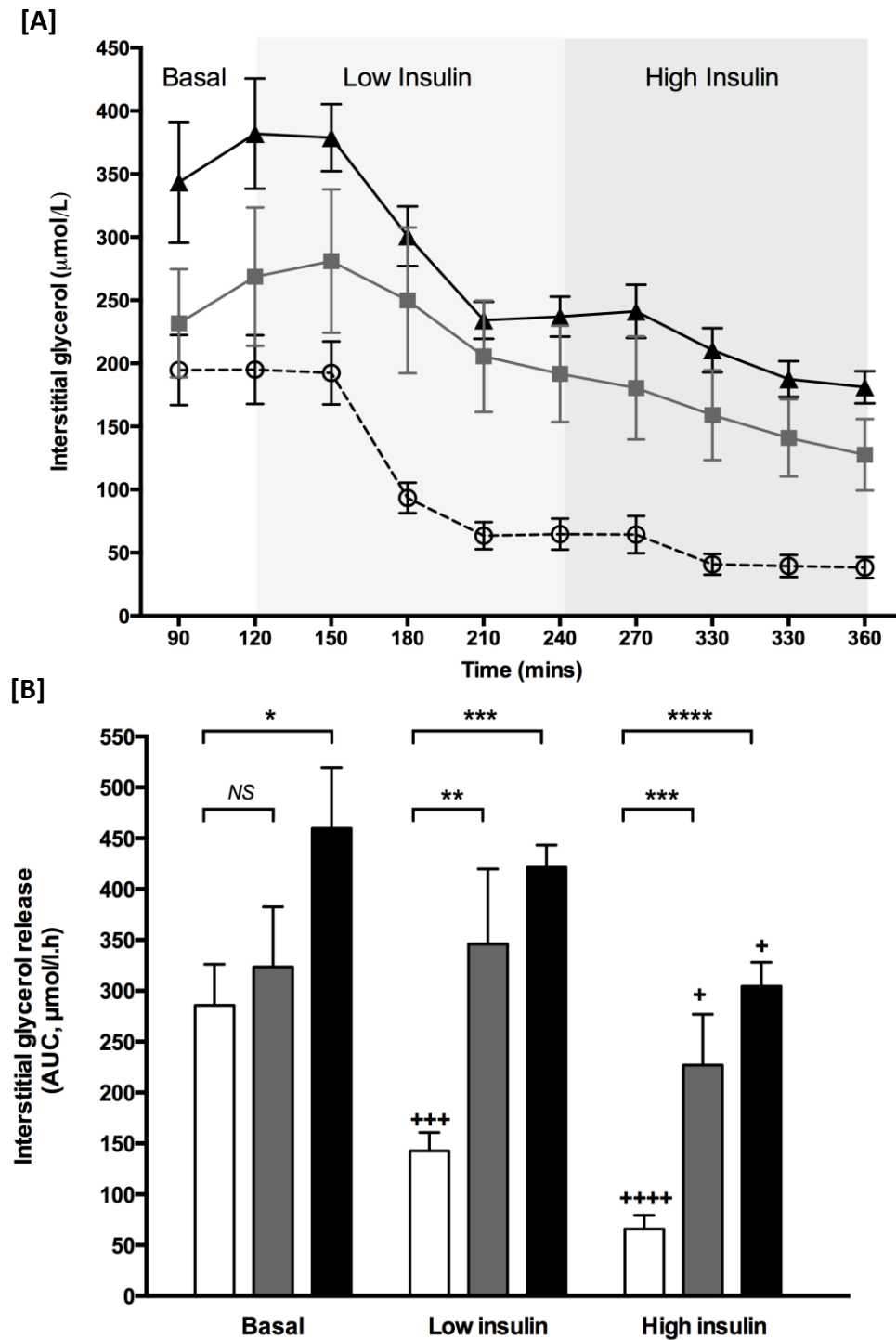
Insulin conc. (pmol/L) ½-maximal suppression of <b>circulating NEFA</b>			Insulin conc. (pmol/L) ½-maximal suppression of sc <b>interstitial adipose glycerol</b>		
Controls (n=15)	NASH (n=16)	p-value	Controls (n=15)	NASH (n=9)*	p-value
65.2 +/- 14.0	226.9 +/- 35.2	0.0003	115.0 +/- 12.3	682.6 +/- 108.3	<0.0001

**Figure 5-7. Subjects with NASH have a disproportionate higher degree of IR in SAT (6-fold vs. controls) compared to whole-body adipose tissue (3-fold vs. controls).**

Line graph representing the concentrations of circulating NEFA (whole-body lipolysis) and interstitial fluid glycerol (SAT-specific lipolysis) in basal, low-dose and high-dose insulin phases of the euglycaemic clamp. Black lines = NASH (mean +/- SE), Grey line = Control. Solid line = glycerol levels, broken line = NEFA levels.

All of the above comparisons remained significant after excluding subjects with type 2 diabetes (n=7) from the NASH cohort (Figure 5-8).

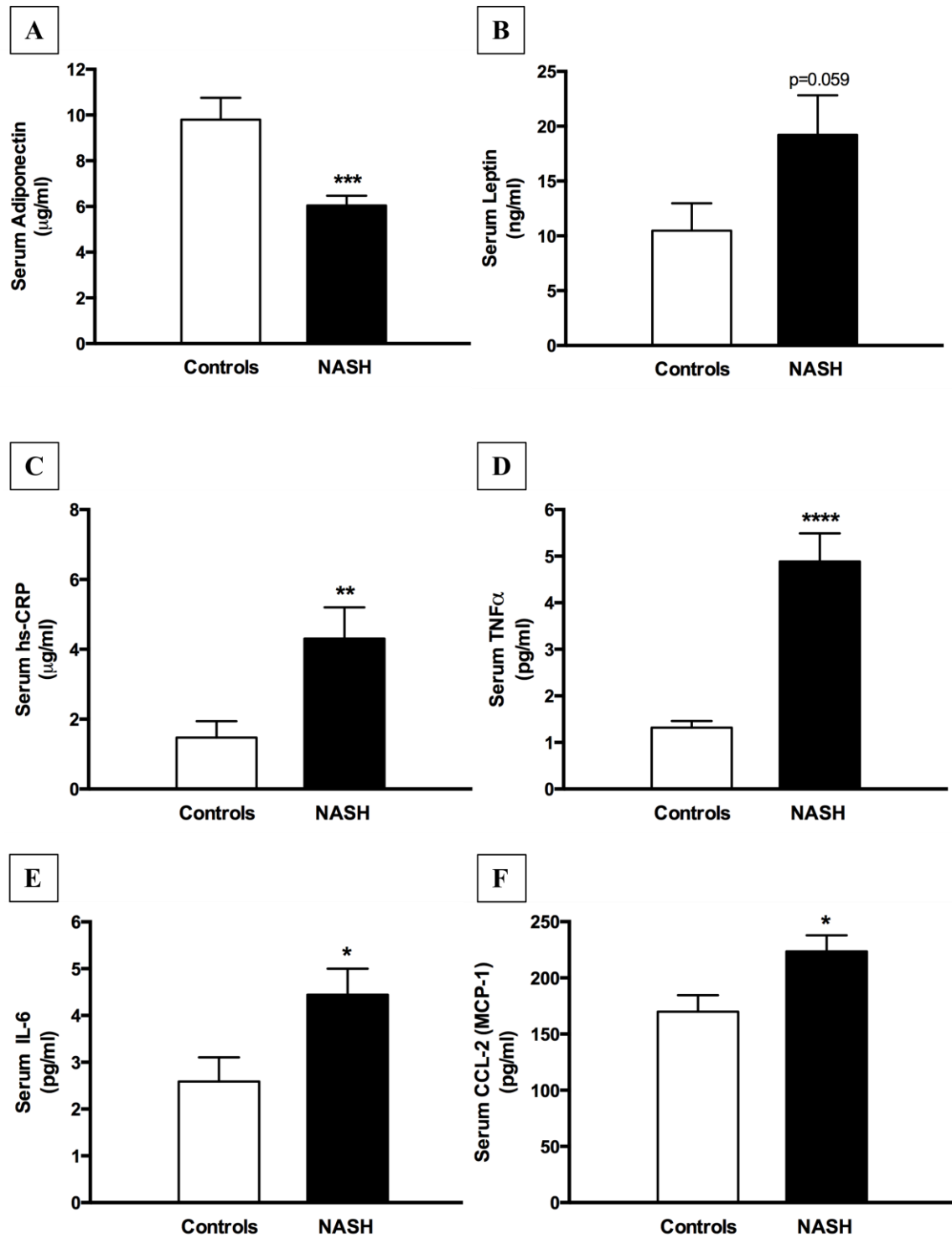




**Figure 5-8. NASH subjects with (n=7)/without type 2 diabetes (n=9) have significant SAT IR.** [A] SAT interstitial fluid concentrations of glycerol during the 2-step hyperinsulinaemic euglycaemic clamp. [B] To determine the rate of lipolysis in SAT under basal and hyperinsulinaemic conditions AUC analysis was performed for interstitial glycerol release. Broken line/White bar = controls, solid grey line/bar = NASH without diabetes, solid black line/bar = NASH with diabetes. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  vs. controls; + $p < 0.05$ , +++ $p < 0.001$ , ++++ $p < 0.0001$  vs. basal phase.

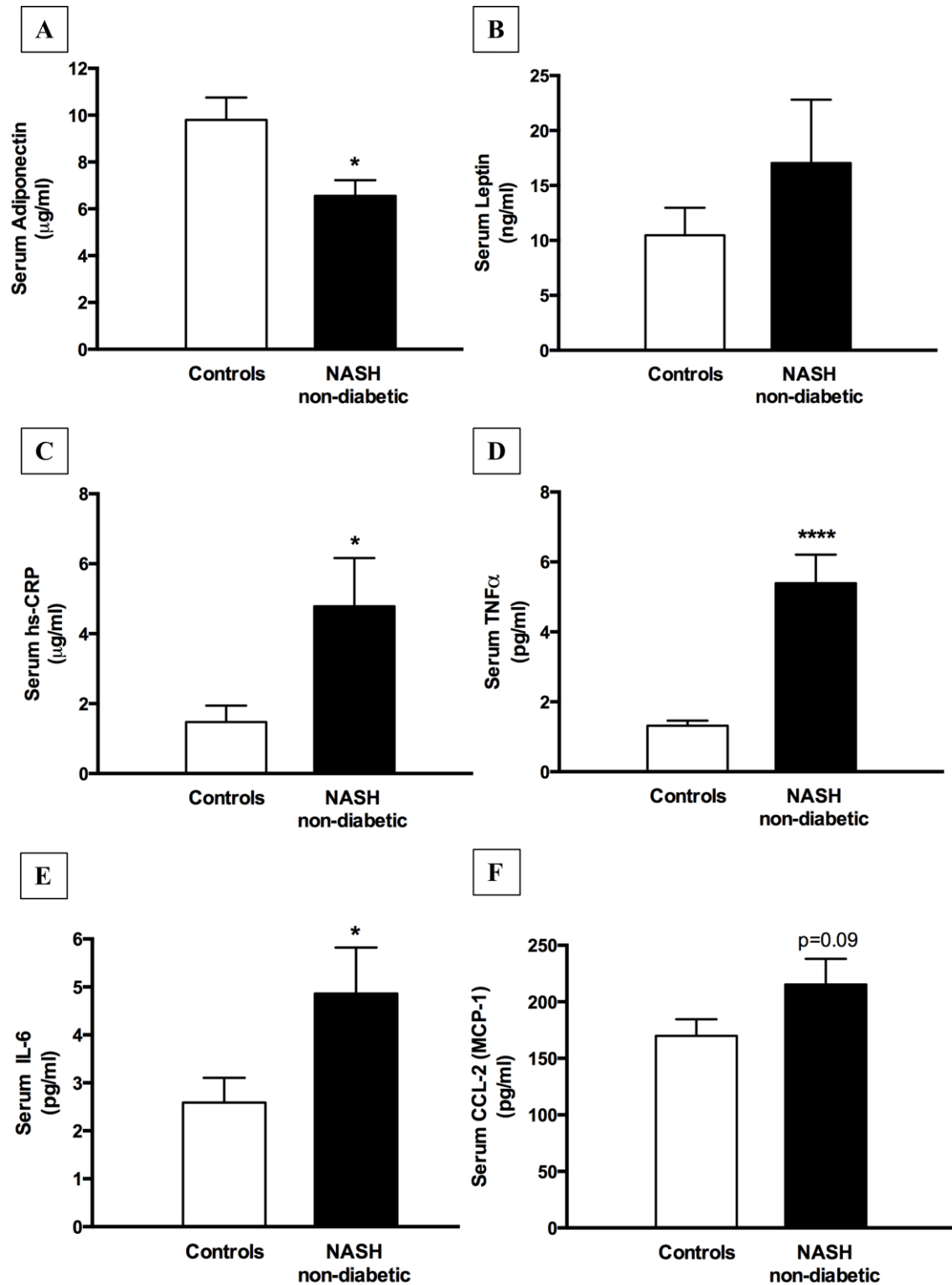
### 5.3.6 Serum adipocytokines and inflammatory cytokines

Subjects with NASH had significantly higher fasting circulating levels of  $\text{TNF}\alpha$  ( $p<0.0001$ ), hs-CRP ( $p<0.05$ ), IL-6 ( $p<0.05$ ) and CCL-2 ( $p<0.05$ ) than controls (Figure 5-9). Serum adiponectin levels ( $p=0.001$ ) were significantly lower in NASH subjects, with a non-significant trend towards higher circulating leptin compared to controls ( $p=0.059$ ). The resultant leptin:adiponectin ratio was 2.5-fold higher in NASH subjects than controls ( $3.22\pm0.5$  vs.  $1.27\pm0.4$ ;  $p=0.0032$ ). There were no significant differences in IL-17, resistin and chemotactic cytokines CCL-3, CCL-4 and CCL-5. With the exception of CCL-2 ( $p=0.09$ ), differences in  $\text{TNF}\alpha$  ( $p<0.0001$ ), hs-CRP ( $p<0.05$ ), IL-6 ( $p<0.05$ ) and adiponectin ( $p<0.05$ ) remained significant after excluding subjects with type 2 diabetes ( $n=7$ ) from the NASH cohort (Figure 5-10).



**Figure 5-9. Fasting adipocytokine profile in subjects with NASH.**

NASH have significantly lower levels of fasting adiponectin [A] and higher levels of pro-inflammatory adipocytokines ([B] leptin, [C] hs-CRP, [D] TNFα, [E] IL-6 and [F] CCL-2/MCP-1. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001 vs. controls.



**Figure 5-10. Fasting adipocytokine profile in non-diabetic subjects with NASH (n=9)**

NASH have significantly lower levels of fasting adiponectin [A] and higher levels of fasting pro-inflammatory adipocytokines ([C] hs-CRP, [D] TNFα, and [E] IL-6). Higher levels of [B] leptin and [F] CCL-2/MCP-1 were seen in non-diabetic subjects with NASH, albeit not achieving significance. \*p<0.05, \*\*\*\*p<0.0001 vs. controls.

## 5.4 Discussion

The data from this study have begun to address the tissue-specific contributions to global IR seen in patients with NASH. Using novel techniques that have functional readouts of insulin-regulated processes in a tissue specific manner allowed an assessment of the contribution of the liver (EGP, DNL), skeletal muscle (Gd), and adipose tissue (circulating NEFA and adipose microdialysis) to systemic IR. By doing so, we have not only demonstrated significant IR at the level of the liver, muscle and adipose tissue, but also by measuring depot-specific glycerol release, our study represents the first *in-vivo* description of dysfunctional abdominal SAT in patients with NASH.

We observed significant levels of hepatic and muscle IR in NASH subjects, as represented by impaired insulin-mediated suppression of hepatic glucose production and impaired insulin-mediated stimulated muscle Gd (weight-adjusted), respectively. In keeping with previous studies (Sanyal et al., 2001; Gastaldelli et al., 2009; Lomonaco et al., 2011), the level of hepatic and muscle IR remained significant when patients with type 2 diabetes were removed from the analysis. Notably, we only saw a non-significant trend towards higher levels of fasting DNL in NASH subjects compared to healthy controls (4.9% vs. 2.8%;  $p=0.16$ ). Even though our low levels of fasting DNL in healthy subjects were consistent with the literature (i.e.  $<5.0\%$ ) (Timlin and Parks, 2005), our findings in NASH are considerably less (4.9% vs. 15-24%) than previously reported (Diraison et al., 2003; Donnelly et al., 2005). This might be attributed to sampling DNL in the fasting state only, oral administration of deuterated water (versus intravenous deuterated tripalmitate (Donnelly et al., 2005)) and/or

the shorter duration of stable isotope labeling compared to previous reports (14 hrs vs. >96 hours (Donnelly et al., 2005)). Due to the nature of the stable isotopes incorporated as part of the clamp and the high rates of labeled glucose infusions required to maintain fasting glycaemia, we were unable to assess the rates of DNL associated with hyperinsulinaemia. It is important to note, however, that Donnelly et al previously reported that the majority of lipid accumulation in NASH was attributed to adipose-derived NEFA (59%), rather than DNL (26%) (Donnelly et al., 2005).

We demonstrated severe adipose tissue dysfunction in patients with NASH using a variety of assessments including adipose IR index, INS-½-max NEFA, adipose tissue microdialysis and circulating adipocytokines. The discrepancy between high fasting leptin and low circulating levels of adiponectin provided further evidence of abnormal adipose tissue function. Indeed, a growing body of evidence indicates that the primary defect in NASH occurs in adipose tissue (Cusi, 2012), from which triglyceride-derived toxic metabolites including the NEFA pool, impair insulin signaling in both skeletal muscle and liver tissue ('lipotoxicity'). A vicious cycle of hepatic, muscle and adipose tissue dysfunction ensues, leading to development of a pathogenic circulating milieu of high levels of insulin, glucose, NEFA and pro-inflammatory cytokines (e.g. hsCRP, IL-6, TNF $\alpha$ , CCL-2), all of which were observed in our patients with NASH. Traditionally, VAT has been recognised as the major contributor to hepatic IR and lipotoxicity (Lebovitz and Banerji, 2005), due to its close proximity to the portal vein and concentration of inflammatory mediators (Fontana et al., 2007; Tordjman et al., 2009). However, as VAT only contributed to 15-20% of circulating NEFA pool (Garg, 2004; Nielsen et al., 2004b), researchers have proposed that either VAT exerted its effects via other non-

NEFA factors including adipocytokines (Lebovitz and Banerji, 2005) or that abdominal SAT plays an important role in lipotoxicity (Miles and Jensen, 2005). Several studies have linked abdominal SAT with IR using euglycaemic clamps in subjects with and without metabolic syndrome (Abate et al., 1995; Abate et al., 1996; Goodpaster et al., 1997; Ferreira et al., 2005), but our data is one of the first to report depot-specific dysfunction in biopsy-proven NASH subjects. Previous studies in NASH patients have solely relied on circulating NEFA to provide estimates of adipose IR (Gastaldelli et al., 2009; Lomonaco et al., 2011; Lomonaco et al., 2012; Musso et al., 2012; Ortiz-Lopez et al., 2012), which are more reflective of whole-body lipolysis, rather than depot-specific (Karpe et al., 2011). By directly measuring interstitial fluid concentrations of glycerol, we report novel insights into the degree of abdominal SAT IR and lipolysis in patients with NASH. The greater magnitude of resistance to the anti-lipolytic effect of insulin in SAT (6-fold vs. controls) in comparison to whole-body adipose (3-fold vs. controls) in our study may well reflect depot-specific IR, in which abdominal SAT is the major source of lipotoxicity in NASH. Interestingly, using paired adipose and liver biopsies from patients undergoing bariatric surgery Tordjman et al have recently shown that deep SAT (and not superficial SAT) has a similar inflammatory profile (i.e. IL-6 gene, macrophage accumulation) to VAT in NASH subjects (Tordjman et al., 2012).

One hypothesis is that abdominal SAT acts as 'buffer' for excess calorific intake and triglyceride deposition. When SAT fails to match the demand, as might be the case in NASH subjects, adipose hypertrophy, inflammation (via monocyte recruitment via CCL-2) and local IR sequentially develop. The resultant localised excess NEFA, as reported here, can result in an overspill of triglyceride-derived toxic metabolites into VAT and subsequently the liver

(Patel and Abate, 2013). Interestingly, the removal of 20% of abdominal SAT in patients with T2DM via liposuction did not improve insulin sensitivity in the liver or adipose tissue (Klein et al., 2004). However, the Klein et al study incorporated no measurements of hepatic inflammation and was of only 10-12 weeks duration. One could argue that this degree of liposuction could alter the buffering mechanism of abdominal SAT and actually have a detrimental effect on lipolysis and the liver over a longer time period.

The role of ethnicity in adipose IR and NASH has recently been investigated, in which Lomonaco and colleagues demonstrated no difference in levels of IR (EGP, fasting NEFA) between Hispanic and Caucasian cohorts with NASH, well-matched for adiposity (Lomonaco et al., 2011). With the exception of two Italian studies (Bugianesi et al., 2005a; Musso et al., 2012), very little data exists in well-characterised patients with NASH of western European descent. Adipose IR index in our UK cohort (9.3 mmol/L.μU/L) was, however, similar to that previously reported in NASH patients from southern Europe and the US (8.0-11.9 mmol/L.μU/L), all of which were 3-6.6 times higher than their respective healthy controls (Gastaldelli et al., 2009; Lomonaco et al., 2012; Musso et al., 2012).

Our study does have limitations, in particular, the metabolic phenotype mismatch between the NASH and 'healthy' controls. This remains a critical challenge in real-world research, due to the high prevalence of obesity and metabolic syndrome in patients with NASH at the time of first presentation. Even though differences in adipose IR remained significant after exclusion of patients with diabetes, we were unable to extrapolate whether our findings were independent of age and measures of adiposity. Gastaldelli et al have reported that



whole-body lipolysis (using adipose-IR index) in NASH is independent of obesity status (defined by BMI) (Gastaldelli et al., 2009), but this requires validation with robust measures of VAT and abdominal SAT mass/volume. In the current study, we were unable to directly compare SAT and VAT, as real-time assessment of dynamic, specific VAT function is not feasible in human studies. The demonstration that SAT is the dominant source of products of triglyceride hydrolysis in healthy humans (Nielsen et al., 2004b) and our finding of marked SAT IR may have significant clinico-pathological implications in patients with NASH. This suggests that it is not simply VAT accumulation that is important in driving the pathological process. Lastly, the cross-sectional design of our study did not allow a causal relationship to be determined between dysfunctional SAT and progressive liver disease. Although warranted, longitudinal study of this kind would be challenging in the context of the chronicity of the disease and the prevalence of metabolic confounders.

In summary, our study highlights that patients with NASH have marked adipose tissue dysfunction, alongside increased hepatic and muscle IR. In particular, we have drawn attention to the profound levels of IR and lipolysis in abdominal SAT, which appear disproportionate to whole-body adipose. Dysfunctional abdominal SAT likely plays a key role in NASH lipotoxicity, rather than being just a bystander to VAT. Whether this is indeed a tissue-mass effect remains to be investigated. Future prospective studies that are sufficiently powered to enable adjustment of metabolic confounders are now required to investigate the relative contribution of SAT (vs. VAT) in disease progression and the impact of novel interventions in NASH.

## **CHAPTER 6: LIRAGLUTIDE REDUCES ADIPOSE INSULIN RESISTANCE AND HEPATIC *DE NOVO* LIPOGENESIS IN NONALCOHOLIC STEATOHEPATITIS: SUB-STUDY RESULTS OF A RANDOMISED-CONTROLLED TRIAL (LEAN).**

### **6.1 Introduction**

In concordance with recent studies ((Sanyal et al., 2001; Gastaldelli et al., 2009; Lomonaco et al., 2011), our in-vivo work has shown that patients with NASH have profound IR at the level of the liver, muscle and in particular, the adipose tissue (Chapter 5). More specifically, we have shown that abdominal SAT exhibits marked IR and undergoes inappropriate excessive lipolysis in patients with NASH. Indeed, it is the overspill and release of triglyceride-derived toxic metabolites, which is now widely thought to be the primary lipotoxic insult in the pathogenesis of NASH and its extra-hepatic complications (Cusi, 2012; Armstrong et al., 2013a). In addition to driving intrinsic hepatic insulin resistance and inflammation, hepatic lipotoxicity is thought to fuel the circulating pro-inflammatory and IR status in NASH, which in turn worsens adipose function and lipolysis in a perpetuating cycle (Cusi, 2012). Therefore, identifying pharmaceutical options that target both adipose insulin resistance and in turn reduce hepatic exposure to the effects of lipotoxic metabolites appears crucial in stopping or reversing the pathogenesis of NASH.

GLP-1 analogues, in particular liraglutide, have been shown to improve glycaemic control, weight loss and most recently liver enzymes in patients with type 2 diabetes (Klonoff et al.,

2008; Armstrong et al., 2013d), thus making them an attractive therapeutic option in NAFLD. Recent animal studies of NAFLD (diet-induced and/or genetic manipulation) have supported these findings by consistently showing improvements in hepatic steatosis with GLP-1 therapy (Samson et al., 2008; Ben-Shlomo et al., 2011; Sharma et al., 2011; Shirakawa et al., 2011; Tomas et al., 2011a; Mells et al., 2012), which in some cases are accompanied by reductions in oxidative stress (Ding et al., 2006; Sharma et al., 2011; Lee et al., 2012) and fibrosis (Trevaskis et al., 2012). Even though there are inconsistencies in the literature (Lee et al., 2007; Parlevliet et al., 2009; Park et al., 2010; Ben-Shlomo et al., 2011; Mells et al., 2012; Parlevliet et al., 2012; Zhang et al., 2013), which are likely due to variations in study design, the majority of murine studies have suggested that these improvements might be due to the effect of GLP-1 on IR. In particular, using established euglycaemic clamp techniques ( $\pm$  isotope tracers), murine studies have shown that chronic GLP-1 administration improves insulin sensitivity by restoration of insulin signalling (i.e. increased Akt activation and/or IRS-1) and reducing hepatic glucose production (Lee et al., 2007; Parlevliet et al., 2009; Park et al., 2010; Mells et al., 2012; Zhang et al., 2013). Similar findings have been reported with short-durations of GLP-1 treatment (i.e. single infusion vs. 6-weeks) in healthy volunteers (D'Alessio et al., 1994; Prigeon et al., 2003) and patients with type 2 diabetes (Gutniak et al., 1992; Zander et al., 2002), but none in the context of NASH. Similarly to the animal experiments, the effect of GLP-1 on muscle insulin sensitivity (i.e. rate of Gd) has been inconsistent in humans, but no studies have reported a detrimental effect (D'Alessio et al., 1994; Orskov et al., 1996; Prigeon et al., 2003). There is a paucity of *in vivo* study regarding GLP-1's effect on adipose IR. *In vitro*, GLP-1 has been shown to increase insulin signaling in adipocytes by upregulation of phosphorylated IRS-1 and Akt, and in turn

enhance insulin-mediated glucose uptake (Egan et al., 1994; Wang et al., 1997; Gao et al., 2007; Vendrell et al., 2011).

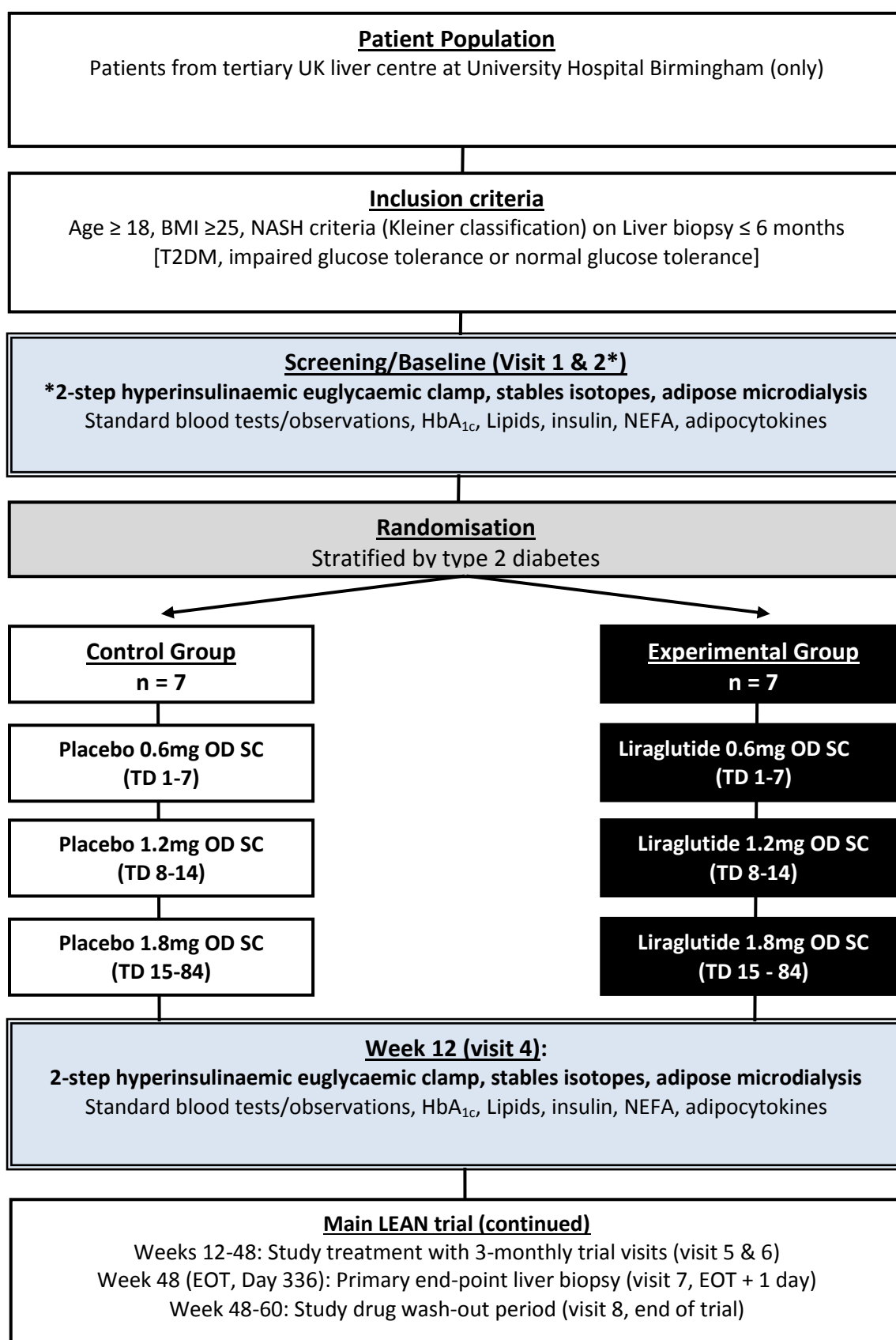
The effect of GLP-1 based therapy on metabolic dysfunction, most notably tissue-specific IR and hepatic lipogenesis, in patients with biopsy-confirmed NASH is currently unknown. In order to investigate such, we incorporated state-of-the-art functional measures of lipid and carbohydrate flux at baseline and 12-weeks into the treatment regimen of the phase II, randomised-control trial (LEAN) trial of the long-acting GLP-1 analogue. The main aims of the metabolic sub-study were to determine the effect of 12-weeks treatment of 1.8 mg liraglutide OD on tissue-specific insulin resistance, hepatic DNL and dysfunctional adipose tissue in patients with biopsy-defined NASH. Patients randomised to placebo-control were used as a benchmark of standard care and for direct comparison to liraglutide.

## **6.2 Methods**

The 12-week mechanistic metabolic sub-study was incorporated into the LEAN trial, which is described in detail in Chapter 3.0. The clinical protocol of the sub-study received full ethical approval from Leicestershire, Northamptonshire & Rutland (ref. 10/H0402/32) Local Research Ethics Committees. All adult subjects gave informed written consent prior to participation in the metabolic sub-study.

### **6.2.1 Study subjects**

Patients who consented and met the eligibility criteria for the double-blinded, randomized, placebo-controlled LEAN trial (Chapter 4) were given the option of participation in the 12-week metabolic mechanistic sub-study. Due to the state-of-the art facilities at the WTCRF (Birmingham) and local expertise (Armstrong/Tomlinson) only patients from the liver unit at the Queen Elizabeth UHB were recruited for the sub-study. In total, 14 adult patients with a definitive diagnosis of NASH on liver biopsy were randomly assigned to 1.8mg OD subcutaneous injections of liraglutide (Victoza®, Novo Nordisk) or placebo-control for 12-weeks (Figure 6-1).



**Figure 6-1. Summary of the mechanistic metabolic study.**

Seven patients were randomised to each treatment groups and underwent paired tests.

### 6.2.2 Study design

At visits 2 (pre-treatment) and 4 (12-weeks) of the LEAN trial, participants underwent paired 2-step hyperinsulinaemic euglycaemic clamps incorporating stable isotopes with concomitant SAT microdialysis at the NIHR Liver Biomedical Research Unit and WTCRF (Birmingham, UK). Detailed descriptions of the metabolic study protocol and data collection are summarised in Chapter 4.

In brief, participants were admitted to the WTCRF the evening (1700 hrs) before the metabolic studies. After a standardised meal, participants were fasted until completion of the metabolic study, with the exception of drinking oral  $^2\text{H}_2\text{O}$  to determine rates of DNL. At 08.00 hours the next morning fasting blood samples were taken and an adipose microdialysis catheter was inserted into the abdominal SAT, prior to starting the 2-step hyperinsulinaemic euglycaemic clamp (*as previously described*). After basal measurements, hepatic and peripheral ('muscle') insulin sensitivity were assessed with consecutive 2hr infusions of insulin at 20 and 100mU/m<sup>2</sup>/min, respectively. Fasting glycaemic concentrations were maintained ('clamped') with a concomitant variable infusion of 20% glucose enriched with U-[<sup>13</sup>C]-glucose (4%) throughout the hyperinsulinaemic phases. During the 6 hour clamp, steady state blood samples were taken at 3 time points in the final 30 minutes of the basal (90-120 min), low-dose (210-240 min) and high-dose insulin (330-360 min) phases (Figure 5-2).

### 6.2.3 Data Collection and Analysis

#### 6.2.3.1 Clinical and biochemical parameters:

Participant demographics and clinical/biochemical measures were recorded at the baseline (visit 2) and 12-week (visit 4) study visits. Systolic/diastolic blood pressure (*average of 2 readings*), waist circumference, weight, height, BMI and bioimpedance (Total body/truncal fat mass) were measured. Fasting blood samples (0800 hours) were analysed for FBC, U&E, LFTs, TSH, lipid profile, HbA1c and plasma glucose using standard laboratory methods (Roche Modular system, Roche Ltd, Lewes, UK). Serum insulin (Mercodia, Sweden), NEFA (Zen-Bio, USA) and adipocytokines (Fluorokine® Multi-Analyte Profiling; R&D Systems, United Kingdom) were measured using commercially available kits, as previously described (Chapter 4). The adipocytokine profiling included adiponectin, leptin, resistin, TNF- $\alpha$ , hs-CRP, IL-6, CCL-2, CCL-4 and CCL-5. In addition, SAT microdialysate samples were analyzed (CMA Iscus Flex, Sweden) for interstitial glycerol concentrations throughout the euglycaemic clamps.

#### 6.2.3.2 Stable Isotope Mass Spectrometry analysis

The enrichment of U-[ $^{13}\text{C}$ ]-glucose in plasma (*for EGP and Gd calculations*) and deuterium ( $^2\text{H}$ ) in the body water pool/ palmitate fraction of total plasma triglycerides (*for DNL calculations*) were determined by gas chromatography-mass spectrometry. The full methods are described in detail Chapter 4.



#### *6.2.3.3 Data definitions and calculations:*

Hepatic (EGP) and muscle (Gd) insulin sensitivity were calculated by using modified versions of the Steele Equations (Steele, 1959; Finegood et al., 1987). The percentage contribution of hepatic DNL to endogenous palmitate synthesis was determined by the incorporation of  $^2\text{H}_2\text{O}$  in the palmitate present in the plasma total triglyceride pool, as previously described in chapter 4.

The rate of whole-body lipolysis was determined by circulating NEFA concentrations in basal phase and in response to hyperinsulinaemic conditions. In order to quantify the whole body adipose tissue insulin sensitivity, the Adipose-IR index and the insulin concentrations causing half-maximal suppression of serum NEFA (INS  $\frac{1}{2}$ -max NEFA) were calculated for each participant using regression analysis. The magnitude of SAT lipolysis in the fasted state and in response to hyperinsulinaemia was determined by the rate of interstitial glycerol release during adipose microdialysis. In order to quantify the degree of SAT insulin sensitivity, the insulin concentrations causing half-maximal suppression of interstitial glycerol (INS- $\frac{1}{2}$ -max glycerol) were calculated for each subject using regression analysis.

#### **6.2.4 Statistical analysis**

Descriptive statistics were applied to characterise the participants randomized to liraglutide and placebo-control. Continuous clinical and laboratory variables are reported as median and IQR as all variables had non-parametric distribution on D'Agostino and Pearson Omnibus

Normality testing. Categorical variables are reported as number and percentages. AUC analysis was performed using the trapezoidal method for interstitial glycerol release during the clamp. Baseline clinical and biochemical characteristics were compared between treatment groups using unpaired Mann-Whitney tests and Fisher exact tests for continuous and categorical variables, respectively. For each treatment group, comparison of baseline versus post-treatment data was performed using paired Wilcoxon signed-rank tests. Unpaired Mann-Whitney tests were used to compare delta change (= Post-treatment – baseline for each subject) of variables in the placebo-control versus liraglutide. The significance level was set at  $p < 0.05$ . All analysis was performed using the GraphPad Prism 5.0 software package.

	Placebo (n=7)	Liraglutide (n=7)	p-value
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## 6.3 Results

### 6.3.1 Study participants

Study participants were recruited to the metabolic sub-study between October 2010 and March 2012. Of the 14 patients recruited, equal numbers were randomised to receive either liraglutide-placebo (referred to as placebo throughout) or liraglutide for 12-weeks. The two treatment groups were well matched with regards to demographic characteristics, clinical and biochemical data (Table 6-1). There were no significant differences between the placebo and liraglutide groups with respect to baseline NASH disease activity (median NAS [IQR]: 4 [3-5] vs. 5 [4-5],  $p<0.05$ ) and the presence of advanced fibrosis (4 vs. 5 participants with Kleiner F3-F4,  $p>0.99$ ).

Importantly, there were no significant differences in the baseline clamp read-outs of organ-specific insulin sensitivity and hepatic DNL between the treatment groups (Table 6-2). With the exception of fasting adiponectin, which was higher in the placebo group (6.02 [5.80-8.68] vs. 4.47 [3.68-6.47]  $\mu\text{g/ml}$ ,  $p<0.05$ ), there were no significant differences in the other serum adipocytokines and inflammatory markers at baseline.

<b>Demographics</b>			
Male sex, n (%)	4 (57.1)	5 (71.4)	1.000
Age (years)	56.0 (39.0-59.0)	59.0 (57.0-60.0)	0.135
Caucasian race, n (%)	7 (100)	7 (100)	1.000
<b>Metabolic parameters</b>			
Type 2 Diabetes, n (%)	3 (42.9)	4 (57.1)	0.478
Impaired glucose tolerance, n (%)	1 (14.3)	2 (28.6)	
Normal glucose tolerance, n (%)	3 (42.9)	1 (14.3)	
HbA1c (%)	5.5 (5.3-6.3)	6.0 (5.6-6.4)	0.244
Pre-study OAD treatment, n (%)	3 (42.9)	5 (71.4)	0.592
BMI (Kg/m <sup>2</sup> )	36.5 (29.3-40.0)	34.0 (30.7-35.9)	0.446
Weight (Kg)	101 (85.6-119)	108 (82.5-115)	0.927
Waist circumference (cm)	118 (99.0-127)	116 (102-121)	0.682
Total fat mass (%)	36.2 (28.0-49.2)	33.9 (30.5-36.7)	0.522
Truncal fat mass (%)	41.1 (30.8-49.2)	34.6 (32.3-36.6)	0.207
Systolic BP (mmHg)	136 (128-146)	128 (121-133)	0.126
Total cholesterol (mmol/L)	4.50 (4.00-5.06)	4.30 (3.90-5.30)	0.925
HDL (mmol/L)	1.15 (1.00-1.38)	1.12 (0.90-1.30)	0.779
LDL (mmol/L)	3.01 (2.21-3.65)	2.58 (2.40-3.86)	0.966
Triglycerides (mmol/L)	1.68 (1.31-2.12)	1.58 (1.43-1.73)	0.644
TSH (μU/L)	2.80 (1.37-4.06)	2.14 (1.31-2.41)	0.689
Creatinine (μmol/L)	62.0 (57.0-77.0)	71.0 (70.0-89.0)	0.300
Platelets (x10 <sup>9</sup> /L)	198 (132-222)	211 (178-219)	0.779
<b>Liver parameters</b>			
AST (IU/L)	49.0 (34.0-70.0)	64.0 (40.0-87.0)	0.596
ALT (IU/L)	57.0 (20.0-70.0)	90.0 (36.0-137)	0.245
GGT (IU/L)	73.0 (55.0-179)	124 (69.0-183)	0.689
ALP (IU/L)	80.0 (56.0-106)	67.0 (57.0-83.0)	0.603
Bilirubin (μmol/L)	14.0 (6.0-19.0)	12.0 (8.0-14.0)	0.555
Albumin (g/L)	46.0 (44.0-49.0)	48.0 (45.0-51.0)	0.470
<b>Liver Histology</b>			
NAS (/8)	4 (3-5)	5 (4-5)	0.355
Kleiner Fibrosis Stage, n (%)			1.000
- 0-2 (mild-moderate)	3 (42.9)	2 (28.5)	
- 3-4 (advanced fibrosis/cirrhosis)	4 (57.1)	5 (71.4)	

**Table 6-1. Demographic, clinical and biochemical characteristics of study participants at baseline.**

Values are median (IQR), unless stated. All blood parameters were in the fasting state. Comparisons of continuous and categorical variables were made with Mann Whitney tests and fisher exact/chi-squared tests, respectively.

	<b>Placebo (n=7)</b>	<b>Liraglutide (n=7)</b>	<b>p-value</b>
<b>Systemic Insulin Sensitivity</b>			

Fasting glucose (mmol/L)	4.51 (4.43-7.17)	5.48 (4.87-5.61)	0.689
Fasting Insulin (pmol/L)	133 (88.0-220)	98.0 (81.9-109)	0.603
<b>Muscle Insulin Sensitivity</b>			
Gd with low-dose insulin (mg/kg/min)	0.68 (0.53-1.27)	0.89 (0.61-0.98)	0.689
Gd with high-dose insulin (mg/kg/min)	3.89 (2.97-4.89)	4.95 (2.49-6.51)	0.872
<b>Hepatic Insulin Sensitivity</b>			
Fasting basal EGP (mg/kg/min)	2.03 (1.63-2.41)	2.14 (1.80-2.27)	0.966
Change EGP with low-dose insulin (mg/kg/min)	-1.00 (-1.34- -0.83)	-0.88 (-1.02- -0.78)	0.207
<b>Hepatic DNL</b>			
% contribution of DNL to palmitate synthesis (%)	5.24 (4.42-6.90)	4.87 (4.38-5.65)	0.376
<b>Adipose Insulin Sensitivity</b>			
Fasting basal NEFA (μmol/L)	421 (397-628)	595 (425-656)	0.966
NEFA with low-dose insulin (μmol/L)	131 (44.6-186)	160 (91.2-204)	0.313
NEFA with high-dose insulin (μmol/L)	15.9 (1.82-36.6)	25.8 (8.51-70.8)	0.689
INS 0.5-MAX NEFA (pmol/L)	180 (106-318)	208 (138-344)	0.872
Adipose-IR index (mmol/L.uU/L)	8.03 (5.34-15.8)	8.42 (5.02-9.88)	0.872
Basal glycerol (AUC μmol/L.h)	382 (215-485)	464 (286-649)	0.446
Glycerol with low-dose insulin (AUC μmol/L.h)	352 (155-437)	454 (347-504)	0.207
Glycerol with high-dose insulin (AUC μmol/L.h)	226 (71.3-372)	307 (214-360)	0.522
<b>Serum Adipocytokines (fasting state)</b>			
Adiponectin (μg/ml)	6.02 (5.80-8.68)	4.47 (3.68-6.47)	<b>0.038</b>
Leptin (ng/ml)	20.4 (8.25-41.1)	12.7 (10.4-22.8)	0.522
Leptin-to-adiponectin ratio (ng /μg)	3.52 (0.95-6.47)	3.15 (2.11-4.24)	0.779
TNFα (pg/ml)	6.49 (1.19-8.39)	3.90 (3.90-6.49)	0.737
Resistin (ng/ml)	6.34 (6.21-7.58)	5.51 (4.71-6.15)	0.053
Hs-CRP (μg/ml)	5.58 (4.49-7.03)	1.55 (0.63-3.89)	0.073
IL-6 (pg/ml)	4.39 (2.83-5.17)	3.61 (2.83-4.00)	0.226
CCL-2 (pg/ml)	229 (183-275)	210 (203-238)	0.872
CCL-4 (pg/ml)	99.5 (60.2-105)	70.8 (58.6-83.6)	0.513
CCL-5 (pg/ml)	54.4 (40.7-72.4)	65.6 (61.0-77.5)	0.207

**Table 6-2. Insulin sensitivity, hepatic DNL and adipocytokine variables of study participants at baseline.**

Values are median (IQR), unless stated. All variables were collected in the fasting state or during the baseline 2-step hyperinsulinaemic euglycaemic clamp, isotope tracers and adipose microdialysis. Comparisons of continuous variables were made with Mann Whitney tests. Key abbreviations: AUC, area under the curve analysis; EGP, endogenous glucose production; Gd, glucose disposal; INS-0.5-MAX NEFA, insulin concentration for ½ maximal suppression of NEFA; TNFα, tumour necrosis factor alpha; hs-CRP, high sensitivity c-reactive protein; IL-6, interleukin 6; CCL, chemokine ligand.

### **6.3.2 Clinical and metabolic variables**

Metabolic/liver variable	Placebo (n=7)	Liraglutide (n=7)	P value **
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12-weeks treatment with 1.8mg liraglutide significantly reduced weight (baseline vs. liraglutide: 108 [82.5-115] vs. 103 [74.9-109] kg;  $p<0.05$ ), BMI (34.0 [30.7-35.9] vs. 32.5 [28.1-33.9];  $p=0.01$ ), and total body fat mass (32.6 [27.6-38.3] vs. 30.8 [24.4-34.5] kg;  $p<0.05$ ) from baseline. Significant reductions from baseline were also seen with liraglutide in markers of central adiposity, including waist circumference (116 [102-121] vs. 110 [100-112] cm;  $p<0.05$ ) and the abdominal fat mass on bioimpedance (19.6 [15.4-22.7] vs. 18.4 [13.1-21.1] kg,  $p<0.05$ ). In contrast, treatment with placebo resulted in no baseline changes with respect to anthropometric measures ( $p>0.1$  in all cases) (Table 6-3). Liraglutide (1.8mg) significant improved glycaemic control (HbA1c %: 6.0 [5.6-6.4] vs. 5.5 [5.5-5.6];  $p<0.05$ ) and total cholesterol (4.3 [3.9-5.3] vs. 3.2 [3.1-4.4] mmol/L;  $p<0.05$ ) from baseline, whereas no improvements were seen in the placebo group. There were no significant improvements from baseline with either treatment in systolic blood pressure, triglycerides and TSH ( $p>0.1$  in all cases). 12-weeks treatment with 1.8mg liraglutide improved liver enzymes from baseline, most notably AST (64 [40-87] vs. 37 [23-39] IU/L;  $p<0.05$ ) and ALT (90 [36-137] vs. 36 [25-74] IU/L;  $p<0.05$ ). In contrast, no significant reductions were seen in the placebo group (Table 6-3).

Direct comparisons (median change from baseline) of the treatment effects of placebo and liraglutide are summarised in Table 6-3. In comparison to placebo, liraglutide significantly reduced weight, BMI, total fat mass, waist circumference, total/LDL cholesterol, creatinine and liver enzymes, namely AST and ALT.

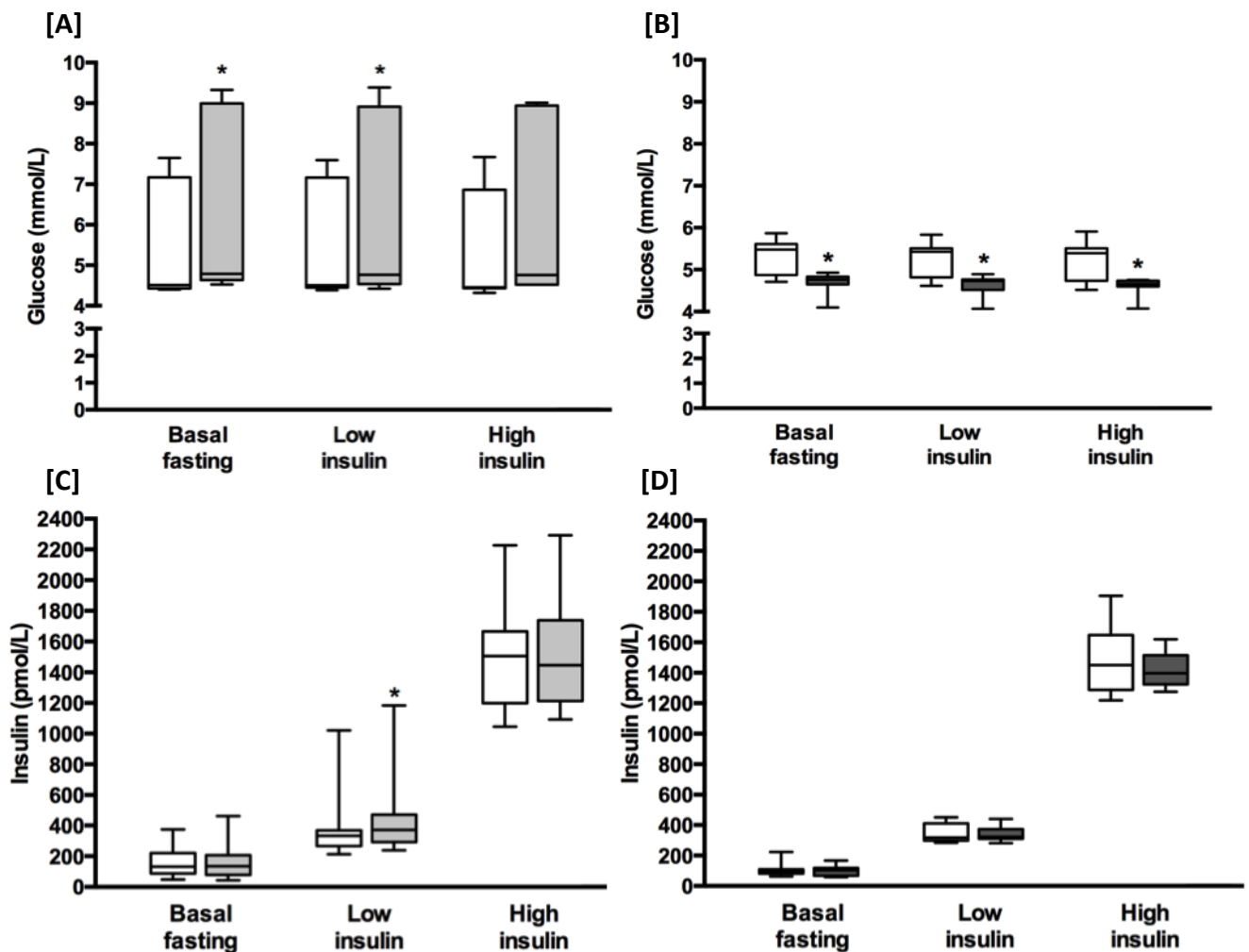
	<b>Median Change from Baseline</b>	<b>P value * (12-wks vs. baseline)</b>	<b>Median Change from Baseline</b>	<b>P value * (12-wks vs. baseline)</b>	
<b>Metabolic factors</b>					
HbA1c %	+0.3 (-0.1 – 1.4)	0.16	-0.3 (-1.2 – -0.1)	<b>0.031</b>	<b>0.006</b>
BMI (Kg/m <sup>2</sup> )	+0.04 (-0.3 – +0.7)	0.81	-1.9 (-2.8 – -1.5)	<b>0.01</b>	<b>0.0006</b>
Weight (Kg)	+0.3 (-1.0 – +2.1)	0.48	-6.0 (-7.0 – -5.0)	<b>0.016</b>	<b>0.0006</b>
Waist circumference (cm)	+2.0 (-3.0 – +2.5)	0.78	-8.0 (-10.0 – -6.0)	<b>0.031</b>	<b>0.004</b>
Total fat mass (%)	-0.4 (-1.1 – +1.3)	>0.99	-3.5 (-4.1 – -1.8)	<b>0.016</b>	<b>0.007</b>
Truncal fat mass (%)	-0.2 (-1.6 – +0.6)	0.38	-1.6 (-2.3 – -0.9)	<b>0.016</b>	0.079
Systolic BP (mmHg)	+5.5 (-5.5 – 10.0)	0.59	-0.5 (-10.0 – -2.5)	0.57	0.40
Total cholesterol (mmol/L)	-0.1 (-0.5 – +0.1)	0.17	-0.8 (-1.2 – -0.5)	<b>0.016</b>	<b>0.004</b>
HDL cholesterol (mmol/L)	-0.2 (-0.2 – -0.1)	<b>0.016</b>	-0.1 (-0.2 – -0.03)	<b>0.031</b>	0.17
LDL cholesterol (mmol/L)	+0.05 (-0.2 – +0.4)	0.81	-0.7 (-0.8 – -0.5)	<b>0.016</b>	<b>0.004</b>
Triglycerides (mmol/L)	+0.3 (-0.04 – +1.0)	0.38	-0.1 (-0.3 – +0.2)	0.38	0.097
TSH (μU/L)	-0.18 (-0.38 – +0.13)	0.47	-0.42 (-0.53 – +0.77)	0.69	0.52
Creatinine (μmol/L)	0.0 (-6.0 – +7.0)	0.63	-7.0 (-13.0 – -3.0)	<b>0.031</b>	<b>0.023</b>
<b>Liver enzymes</b>					
AST (IU/L)	+9.0 (-3.00 – +15.0)	0.36	-27.0 (-45.0 – -3.0)	<b>0.016</b>	<b>0.025</b>
ALT (IU/L)	-4.00 (-6.00 – +16.0)	0.22	-54.0 (-65.0 – -18.0)	<b>0.031</b>	<b>0.004</b>
GGT (IU/L)	-11.0 (-31.0 – -4.0)	0.22	-36.0 (-75.0 – -25.0)	<b>0.016</b>	0.079
ALP (IU/L)	-8.3 (-18.7 – -2.0)	0.156	-9.8 (-14.0 – -7.0)	<b>0.031</b>	0.60

**Table 6-3. Changes in metabolic and liver parameters in participants receiving liraglutide and placebo.**

Values are median (IQR). All blood parameters were in the fasting state. \*Baseline comparisons (for each treatment) were made with paired Wilcoxon-signed rank tests and \*\*comparisons of liraglutide vs. placebo unpaired Mann Whitney tests were used.

### **6.3.3 Systemic insulin sensitivity**

1.8mg liraglutide significantly reduced fasting serum glucose from baseline (5.48 [4.87-5.61] vs. 4.76 [4.65-4.83] mmol/L;  $p<0.05$ ), but had no effect on fasting insulin levels (98.0 [81.9-109] vs. 103 [67.5-118] pmol/L;  $p=0.81$ ), consistent with decreased systemic insulin resistance (Figure 6-2B&D). In contrast, fasting serum glucose increased in patients receiving placebo despite no change in fasting hyperinsulinaemia (Figure 6-2A&C).

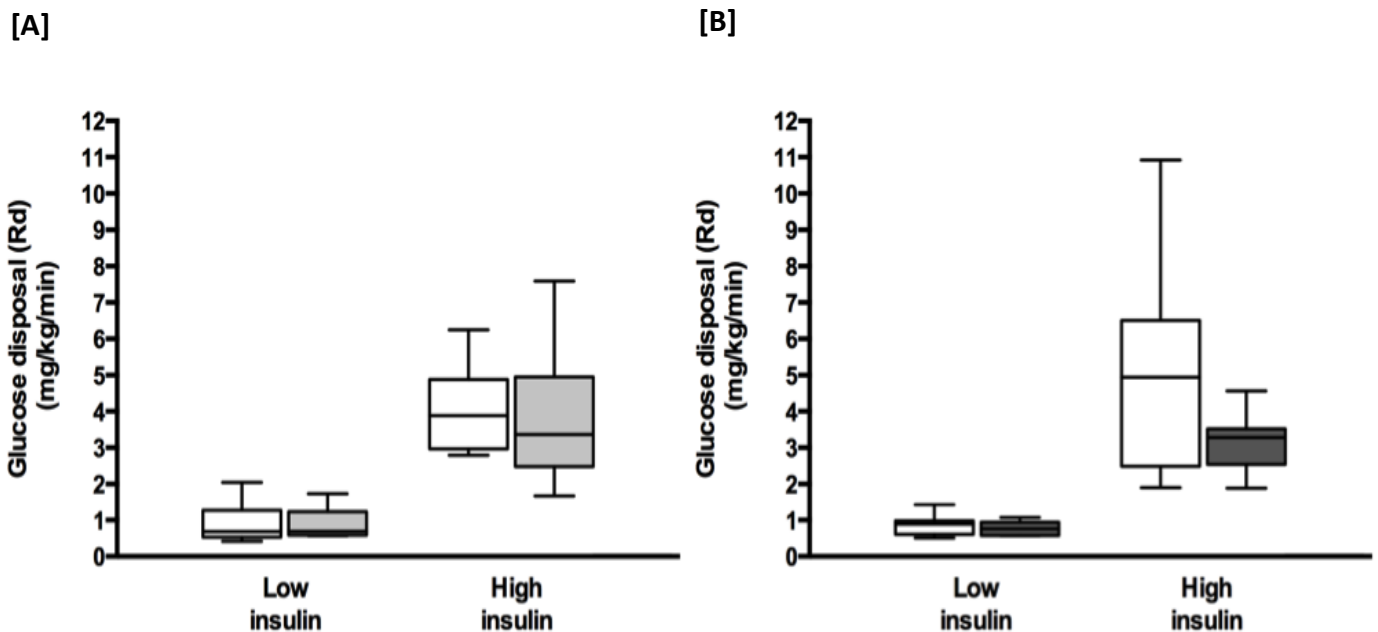


**Liraglutide (*right*) significantly decreases fasting serum glucose with no change in serum insulin, representing increased systemic insulin sensitivity.**

Tukey box-and-whisker plots of fasting glucose ([A] placebo, [B] liraglutide) and insulin ([C] placebo, [D] liraglutide) concentrations at basal and hyperinsulinaemic phases of euglycaemic clamp. Key: White bar = baseline, light grey bar = placebo, dark grey bar = liraglutide. \* $p<0.05$  vs. basal phase.



Patients treated with liraglutide had significant improvements in fasting glucose compared to those receiving placebo (median baseline change: -0.65 [-0.91–0.17] vs. +0.28 [+0.01–1.34] mmol/L;  $p<0.001$ ). There was no significant difference between treatment groups with respect to changes in fasting insulin levels (median baseline change: +3.46 [-37.9–19.9] vs. -3.30 [-13.5–30.3] pmol/L;  $p=0.88$ ). 1.8mg liraglutide had no significant effect on the weight-adjusted Gd rates in the low-dose (0.89 [0.61-0.97] vs. 0.76 [0.57-0.94] mg/kg/min;  $p=0.69$ ) and high-dose insulin phases of the clamp (4.95 [2.49-6.50] vs. 3.28 [2.54-3.51] mg/kg/min;  $p=0.38$ ). Similarly, patients treated with placebo had no differences in weight-adjusted Rd rates from baseline (Figure 6-3).



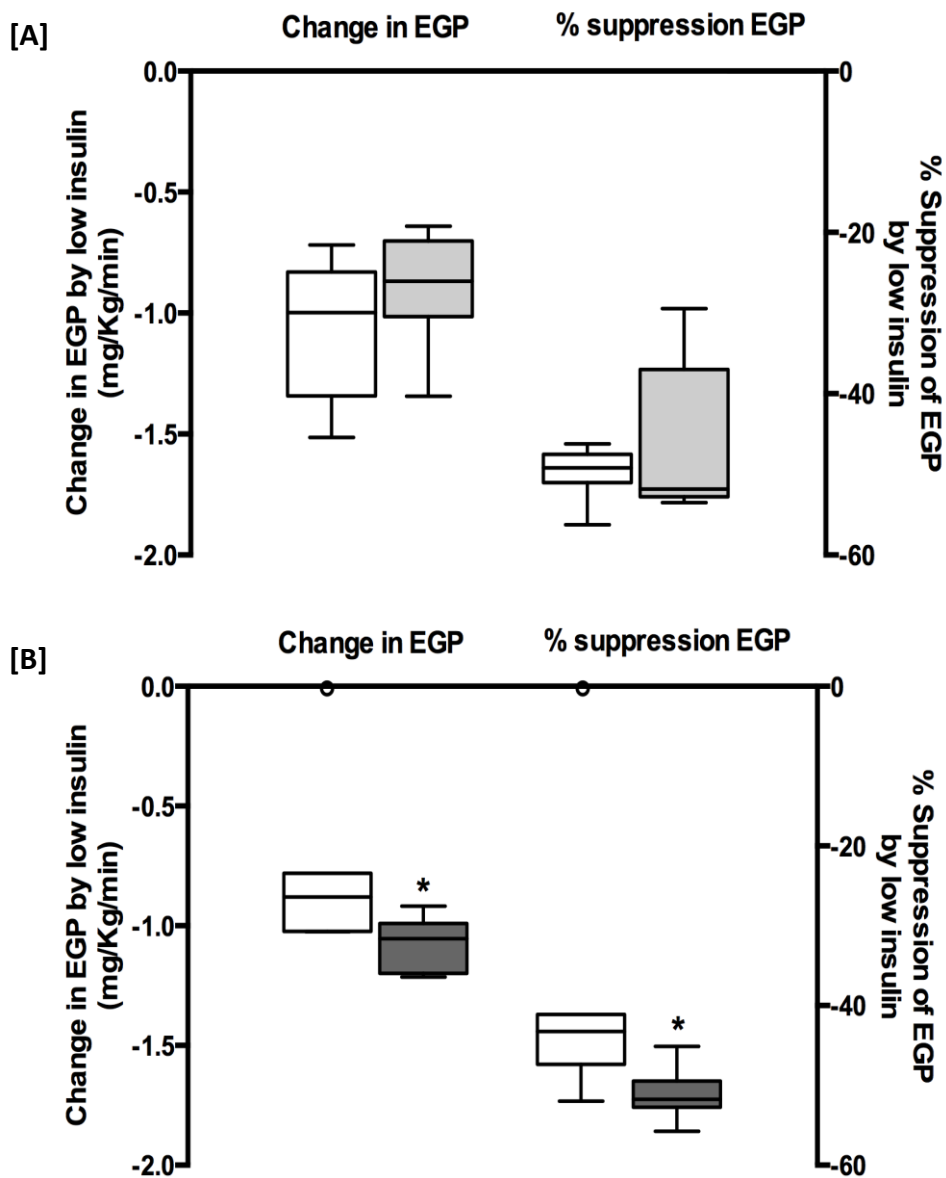
**Figure 6-3. Liraglutide (*right*) has no significant effect on muscle insulin sensitivity.**

Tukey box-and-whisker plots of weight-adjusted glucose disposal (Rd) rates at low- and high-dose insulin phases of the euglycaemic clamp. 12-weeks treatment with placebo [A] or liraglutide [B] did not significantly improved Rd (muscle insulin sensitivity) in patients with NASH. Key: White bar = baseline, light grey bar = placebo, dark grey bar = liraglutide.

There was no significant difference between treatment groups with respect to changes in Rd rate from pre-treatment levels (median baseline change: liraglutide -0.04 [-0.41—+0.18] vs. placebo +0.15 [-0.31—+0.19] mg/kg/min;  $p=0.60$ ). Together, this means that 1.8mg liraglutide did not affect muscle insulin sensitivity in patients with NASH.

#### **6.3.4 Hepatic insulin sensitivity**

1.8mg liraglutide significantly increased the percentage suppression of hepatic EGP with low-dose insulin (-43.2 [-47.4—-41.1] vs. -51.7 [52.7—49.5] %;  $p<0.05$ ), consistent with decreased hepatic insulin resistance (Figure 6-4). Treatment with placebo resulted in no significant change in EGP (-49.2 [-51.0—-47.5] vs. -51.9 [52.8—37.0] %;  $p=0.94$ ). Overall, the median change in percentage suppression of hepatic EGP with low dose insulin was significantly greater with liraglutide than placebo (-9.36 [-11.6—-2.12] vs. -2.54 [-4.33—+16.8] %;  $p<0.05$ ).

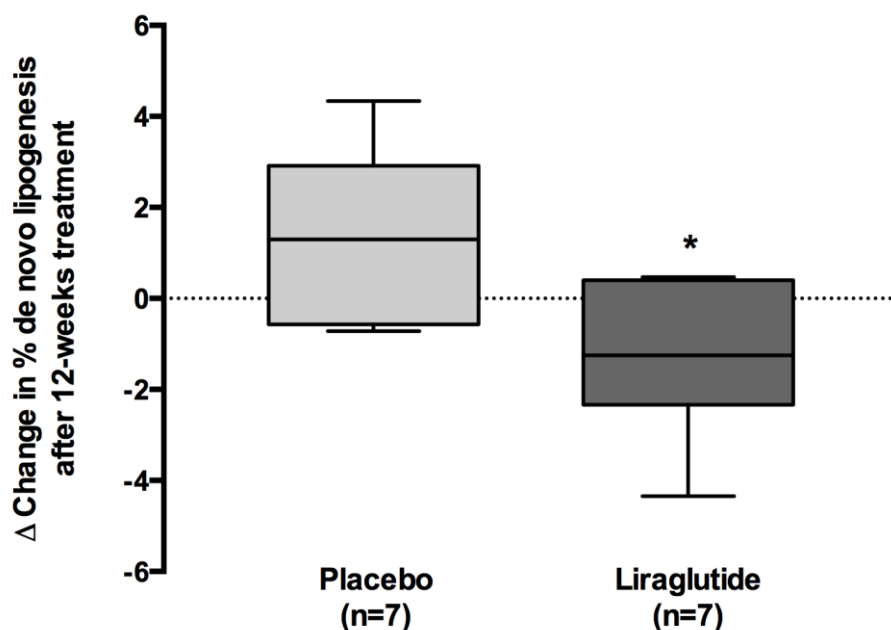


**Figure 6-4. Liraglutide significantly reduces hepatic insulin resistance**

Tukey box-and-whisker plots highlight that liraglutide [B] significantly increased the suppression of EGP with low dose insulin, representing increased hepatic insulin sensitivity. No differences were seen in the placebo [A] group. Key: White bar = baseline, light grey bar = placebo, dark grey bar = liraglutide. \* $p < 0.05$  treatment vs. baseline.

### 6.3.5 Hepatic DNL

The percentage contribution of hepatic DNL to total endogenous palmitate synthesis did not significantly change from baseline with either placebo (5.24 [4.42-6.90] vs. 6.85 [4.43-9.58] %;  $p=0.22$ ) or liraglutide (4.87 [4.38-5.66] vs. 3.26 [2.58-4.85] %;  $p=0.15$ ) (Figure 6-5). However, liraglutide significantly reduced hepatic DNL when compared directly to placebo (median change: -1.26 [-2.34--0.40] vs. +1.30 [-0.56--2.91] %;  $p<0.05$ ).

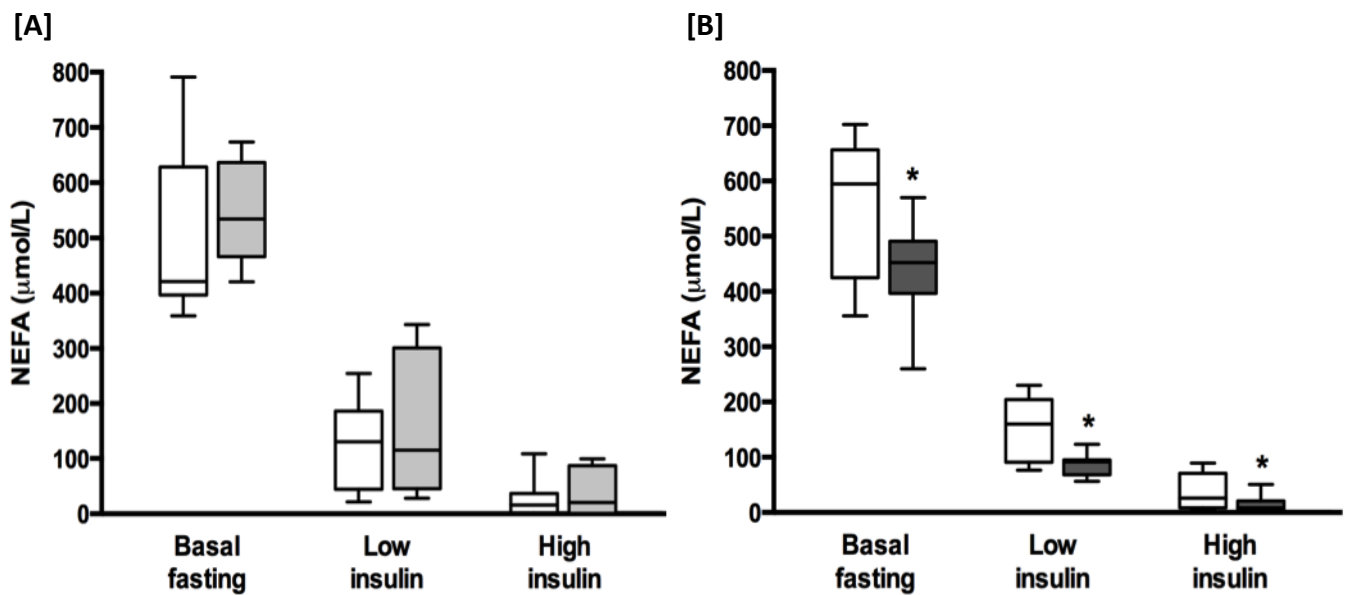


**Figure 6-5. Liraglutide significantly reduces hepatic DNL compared to placebo.**

Hepatic DNL measured via  $^2\text{H}_2\text{O}$  incorporation into palmitate synthesis. Key: light grey bar = placebo, dark grey bar = liraglutide. \* $p<0.05$  vs. placebo.

### 6.3.6 Adipose insulin sensitivity and lipolysis

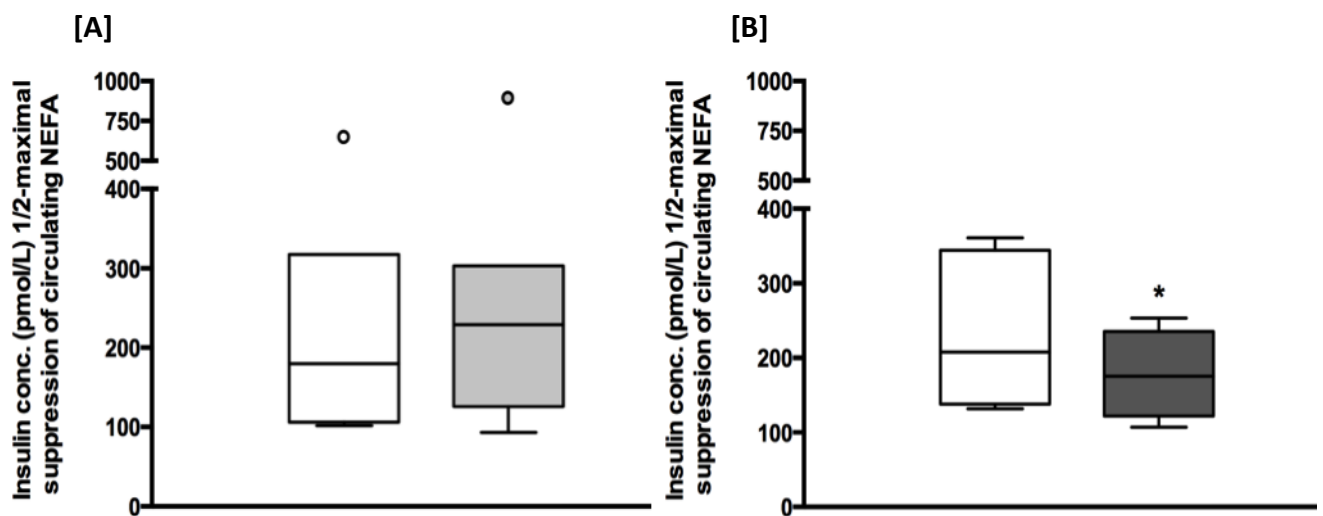
Liraglutide significantly reduced circulating NEFA in the fasting (595 [425-656] vs. 452 [397-491]  $\mu\text{mol/L}$ ;  $p<0.05$ ) and hyperinsulinaemic states, representing decreased whole-body lipolysis. In contrast, no differences were reported in the fasting (421 [397-628] vs. 534 [466-636]  $\mu\text{mol/L}$ ;  $p=0.35$ ) and hyperinsulinaemic states in patients who received placebo (Figure 6-6). Circulating NEFA significantly improved with liraglutide versus placebo treatment in the fasting (-95.8 [-183--79.8] vs. +61.8 [-122--164]  $\mu\text{mol/L}$ ;  $p<0.05$ ), low-dose insulin (-66.8 [-115--34.5] vs. +1.05 [-33.7--88.7]  $\mu\text{mol/L}$ ;  $p=0.007$ ) and high-dose insulin (-15.1 [-23.6--4.1] vs. 0.0 [-5.52--16.1]  $\mu\text{mol/L}$ ;  $p=0.007$ ) phases of the euglycaemic clamps.



**Figure 6-6. Liraglutide significant reduces circulating NEFA (whole-body lipolysis).**

Tukey box-and-whisker plots representing NEFA concentrations at the basal and hyperinsulinaemic phases of euglycaemic clamp. Liraglutide [B] reduced NEFA at every phase of the clamp, whereas placebo [A] remained no different. Key: White bar = baseline, light grey bar = placebo, dark grey bar = liraglutide. \* $p<0.05$  treatment vs. baseline.

In order to determine the effect on whole-body adipose insulin sensitivity, Adipose-IR index (fasting state) and the insulin concentration required to cause half-maximal suppression of serum NEFA (INS-½-max NEFA) were calculated pre/post treatment for each participant. In the fasting state, liraglutide significantly reduced the Adipose-IR index (8.42 [5.02-9.88] vs. 6.26 [4.41-7.28] mmol/L.uU/L;  $p<0.05$ ), representing decreased adipose IR, whereas no significant change occurred with placebo (8.03 [5.34-15.8] vs. 10.2 [7.67-12.5] mmol/L.uU/L;  $p=0.38$ ). Similarly, liraglutide significantly reduced INS-½-max NEFA, with no reported change with placebo (Figure 6-7). Furthermore, liraglutide significantly INS-½-max NEFA when directly compared to placebo (-24.9 [-107—9.73] vs. +54.8 [-14.4—+56.0] pmol/L;  $p<0.05$ ), consistent with increased anti-lipolytic action of insulin with liraglutide.

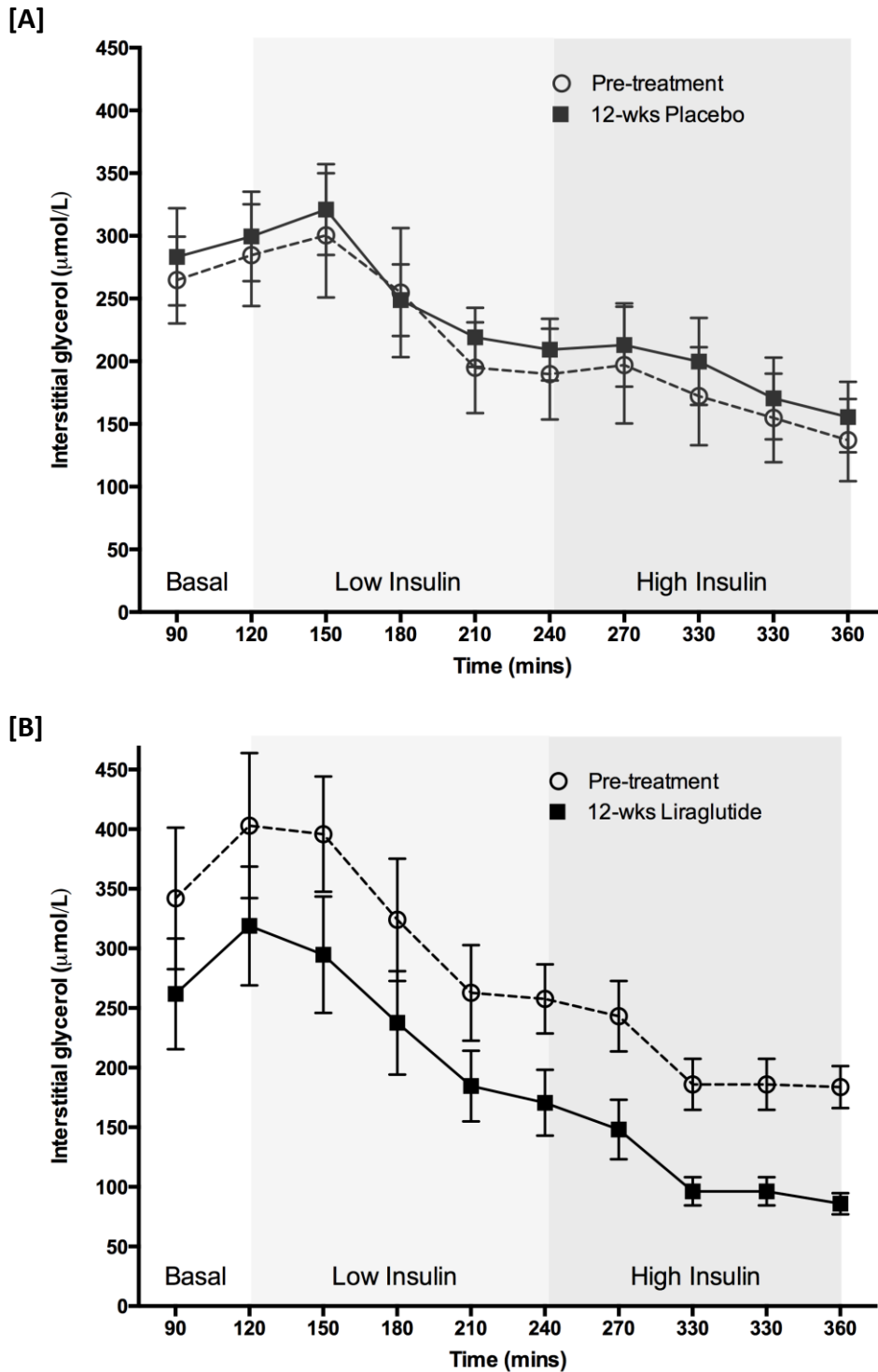


**Figure 6-7. Liraglutide significant reduces whole-body adipose IR.**

Tukey box-and-whisker plots representing the effect of placebo [A] and liraglutide [B] on INS-½-max NEFA. Key: White bar = baseline, light grey bar = placebo, dark grey bar = liraglutide. Circles represent outliers defined as 3<sup>rd</sup> quartile + 1.5×IQR. \* $p<0.05$  treatment vs. baseline.

Liraglutide decreased SAT lipolysis, as demonstrated by a reduction in interstitial fluid glycerol concentrations at all hyperinsulinaemic time-points of the euglycaemic clamp (Figure 6-8B). In contrast there were differences in glycerol concentrations between baseline and after 12-weeks of placebo (Figure 6-8C). AUC analysis was performed to analyse the rate of glycerol release during the three phases of the euglycaemic camp (basal, low- and high-dose insulin).

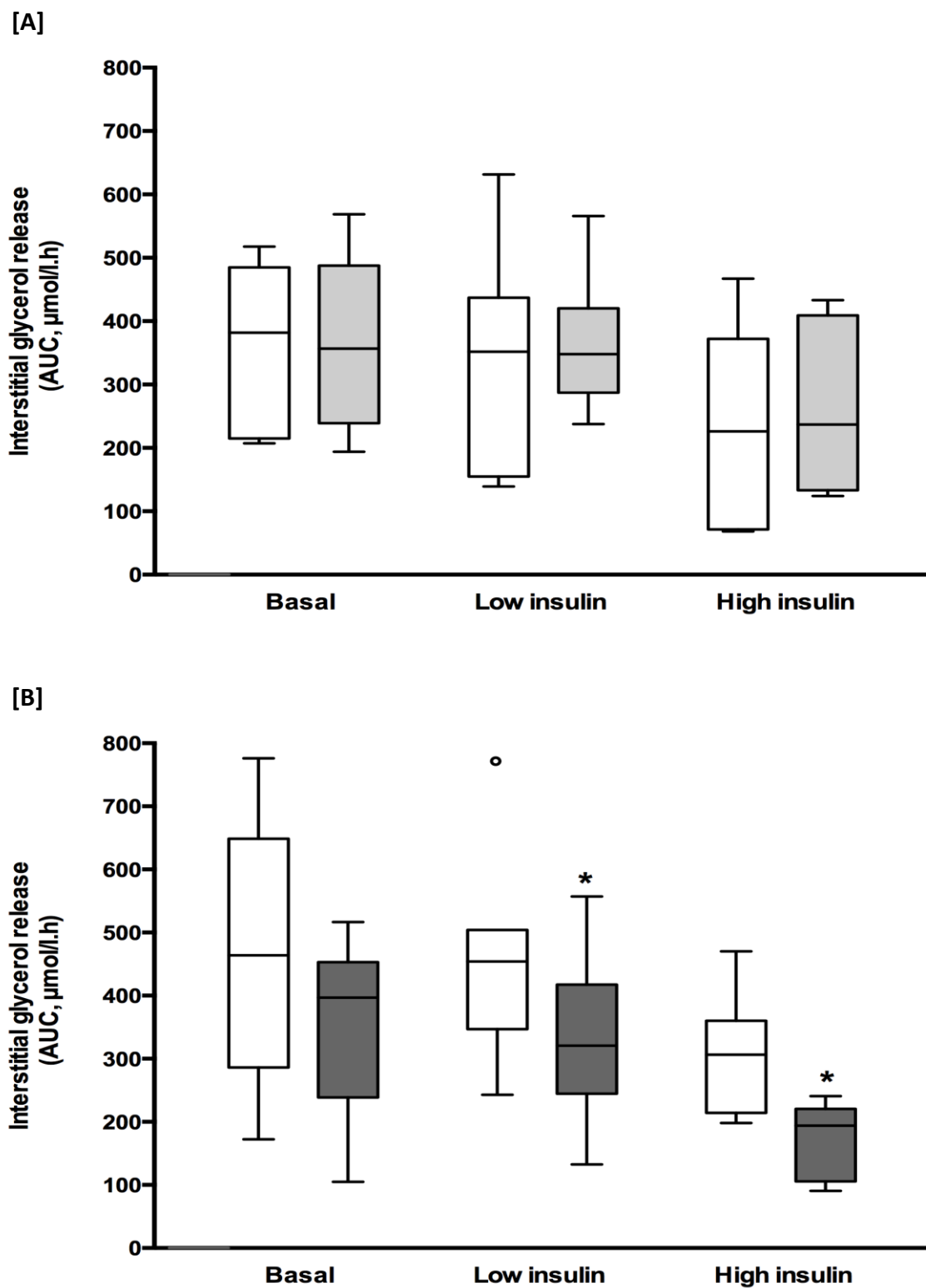
Liraglutide significantly reduced the rate of glycerol release from SAT in response to both low-dose (454 [347-504] vs. 321 [245-418] AUC.μmol/L.hr;  $p<0.05$ ) and high-dose insulin (307 [214-360] vs. 194 [106-221] AUC.μmol/L.hr;  $p<0.05$ ), representing decreased abdominal SAT insulin resistance (Figure 6-9). In contrast, treatment with placebo had no effect on the rate of glycerol release into the interstitial fluid at both low-dose (352 [155-437] vs. 348 [287-420] AUC.μmol/L.hr;  $p=0.68$ ) and high-dose insulin (226 [71.3-372] vs. 237 [133-409] AUC.μmol/L.hr;  $p=0.11$ ).



**Figure 6-8. Liraglutide reduces abdominal SAT lipolysis.**

Line graphs represent the mean $\pm$ SE concentrations of glycerol in the interstitial fluid, measured from the SAT of each participant using in situ microdialysis throughout the 6-hr euglycaemic clamp. **[A]** Liraglutide decreased glycerol release from baseline (pre-treatment), whereas there were no clear differences after placebo treatment **[B]**.





**Figure 6-9. Liraglutide reduces abdominal SAT IR.**

Area under the curve analysis: Liraglutide significantly reduced glycerol release from SAT in response to both low-dose and high-dose insulin [B], representing decreased abdominal SAT IR. No changes were seen with placebo group [A]. \* $p < 0.05$  12-wk treatment vs. baseline.

### 6.3.7 Serum adipocytokines and inflammatory mediators

There were no significant changes in the serum adipocytokine and inflammatory profile in patients who received placebo Table 6-4. In contrast, liraglutide significantly reduced fasting serum leptin (12.7 [10.4-22.8] vs. 10.6 [8.39-13.5] ng/ml;  $p<0.05$ ) and increased adiponectin (4.47 [3.68-6.47] vs. 6.28 [4.24-8.75]  $\mu$ g/ml;  $p<0.05$ ) from baseline. This reciprocal change was exemplified by a 50% reduction in the leptin-to-adiponectin ratio (3.15 [2.11-4.24] vs. 1.55 [1.18-2.85] ng/ $\mu$ g;  $p<0.05$ ), implying improved adipose metabolism. Liraglutide significantly reduced the levels of well-recognised pro-inflammatory markers, namely CCL-2 (210 [203-238] vs. 203 [171-225] pg/ml;  $p<0.05$ ) and hs-CRP (1.55 [0.63-3.89] vs. 0.46 [0.25-1.53]  $\mu$ g/ml;  $p<0.05$ ), but increased resistin levels (5.52 [4.71-6.15] vs. 6.15 [5.04-7.79] ng/ml;  $p<0.05$ ). In direct comparison to the placebo group, liraglutide significantly improved circulating of adiponectin, leptin and CCL-2, which are all key features of adipose inflammation and dysfunction.

Cytokine/ inflammatory marker	Placebo (n=7)		Liraglutide (n=7)		P value **  Liraglutide Vs. placebo
	Median change	P value * (12-wks vs. baseline)	Median Change	P value * (12-wks vs. baseline)	
Adiponectin (µg/ml)	-0.22 (-1.67 – 0.38)	0.58	+1.33 (0.56 – 1.86)	<b>0.016</b>	<b>0.018</b>
Leptin (ng/ml)	+0.52 (-1.83 – 0.76)	0.69	-3.16 (-3.56 – -1.98)	<b>0.016</b>	<b>0.026</b>
LAR (ng/µg)	+0.045 (-1.48 – 0.55)	0.79	-1.04 (-1.91 – -0.85)	<b>0.016</b>	0.097
Resistin (ng/ml)	-0.025 (-0.76 – 0.48)	0.94	+0.59 (0.46 – 1.04)	<b>0.016</b>	0.073
TNFα (pg/ml)	0.00 (-0.12 – 1.33)	0.81	-1.33 (-2.72 – 0.00)	0.13	0.12
hs-CRP (µg/ml)	-1.48 (-3.47 – 2.29)	0.47	-0.45 (-1.23 – -0.22)	<b>0.016</b>	0.78
IL-6 (pg/ml)	+0.04 (-1.56 – 1.95)	0.92	-0.78 (-1.17 – 0.00)	0.16	0.55
CCL-2 (pg/ml)	+19.2 (-8.3 – 30.1)	0.16	-9.14 (-22.3 – -6.29)	<b>0.031</b>	<b>0.026</b>
CCL-4 (pg/ml)	-48.6 (-50.7 – 8.91)	0.38	-40.4 (-63.6 – 2.41)	0.11	0.60
CCL-5 (pg/ml)	-3.20 (-7.08 – 15.8)	0.99	+4.58 (-8.07 – 13.2)	0.38	0.87

**Table 6-4. Effect of liraglutide and placebo on fasting serum adipocytokines and inflammatory markers.**

Adipocytokine profile performed on fasting serum at baseline and after 12-weeks treatment with either placebo or liraglutide. Fluorokine MAP and luminex technology were used to analyse the samples. \*p-value, wilcoxon pairs-signed rank test. \*\*p-value, unpaired Mann Whitney U test. Key: CCL, chemokine ligand; hs-CRP, high sensitivity c-reactive protein; IL, interleukin; LAR, leptin-adiponectin ratio; TNFα, tumour necrosis factor alpha.

## 6.4 Discussion

Adipose tissue IR and lipotoxicity are key pathognomonic features in NASH. Therefore, therapies that rescue the liver from such metabolic insults are essential for preventing the progression of NASH to end-stage disease (Cusi, 2012). Our prospective, randomised-control study highlights that 1.8mg liraglutide improves several key clinical and metabolic features of NASH, including central adiposity, hyperlipidaemia and glycaemic control. Using state-of-the-art measures of carbohydrate and lipid flux, our study represents the first *in vivo* description of the beneficial effects of liraglutide on hepatic lipogenesis ( $\downarrow$ DNL), hepatic IR ( $\downarrow$ EGP), and most notably adipose IR ( $\downarrow$  circulating NEFA,  $\uparrow$  adiponectin) and inflammation ( $\uparrow$  adiponectin,  $\downarrow$  CCL-2) in patients with NASH. By suppressing glycerol release from abdominal SAT, we found that liraglutide potentially has depot-specific effects, which likely play a significant role in reducing lipotoxic injury in patients with NASH.

Liraglutide significantly improved glycaemic control in the patients with NASH, as seen by decreased fasting glucose levels and HbA1c. The fact that this occurred in the absence of changes in fasting insulin reflects an overall improvement in systemic IR. This effect did not appear to be attributed to changes in muscle insulin sensitivity, as liraglutide had no significant effect on insulin-mediated muscle glucose uptake (Gd) at low or high doses of insulin. Whether the lack of the GLP-1 effect on muscle IR was specific to patients with NASH or due to our experimental design is unclear. One possible explanation is that the high dose of insulin ( $100\text{mU}/\text{m}^2/\text{min}$ ) might have saturated the insulin-mediated glucose uptake in the muscle of our NASH cohort, thereby masking any subtle insulin sensitising changes that

might have occurred with liraglutide. The high dose of insulin, which transpired to be supra-physiological serum concentrations (mean serum 1478 pmol/L), was used to ensure total suppression of hepatic EGP in a state of marked IR. Furthermore, even though muscle is the main site of insulin-mediated glucose uptake in the body, a limitation of the second-phase of the hyperinsulinaemic clamp technique is that it can't distinguish between muscle and adipose glucose uptake. Even though there are no other studies addressing the effect of GLP-1 on Gd in patients with NASH, animal models (Lee et al., 2007; Parlevliet et al., 2009; Ben-Shlomo et al., 2011; Zhang et al., 2013) and reports in non-diabetic/diabetic individuals on GLP-1 based treatments are inconsistent. Earlier studies in humans (Orskov et al., 1996; Toft-Nielson et al., 1996; Vella et al., 2002), the majority of which were in healthy volunteers, failed to show an effect on muscle Gd with short or chronic infusions of GLP-1 during hyperinsulinaemia. Subsequent studies, however, have reported both insulin-mediated (Zheng et al., 2009; Park et al., 2010; Zhang et al., 2013) and insulin-independent effects of GLP-1 on the promotion of Gd in muscle (Meneilly et al., 2001; Egan et al., 2002). The latter finding, however, is not uniform amongst human studies (Seghieri et al., 2013).

By incorporating glucose isotope tracers in the euglycaemic clamp, we have previously shown that the normal suppression of hepatic EGP with insulin (mean serum insulin 330 pmol/L) is blunted in patients with NASH (Chapter 5). In contrast to unmodified muscle IR, we found that liraglutide significantly increased insulin-mediated suppression of EGP compared to placebo. This increase in hepatic insulin sensitivity, together with the reductions in the circulating NEFA pool, are likely the major determinants of improved fasting glycaemia and systemic insulin sensitivity in NASH patients with liraglutide. Our

findings are supported by euglycaemic clamp studies in rodent models of NAFLD (ALIOS-induced (Mells et al., 2012), high fat diet +/- adiponectin knockdown (Parlevliet et al., 2009; Zhang et al., 2013)), and patients with (Gutniak et al., 1992; Zander et al., 2002) and without (D'Alessio et al., 1994; Prigeon et al., 2003) type 2 diabetes, all of which underwent either a single or prolonged (max. 8 weeks (Zhang et al., 2013)) infusion of active GLP-1. Prior to our study, no human studies had characterised the underlying liver status of the participants, nor had they determined the effect of hepatic EGP with a self-administered, long-acting GLP-1 therapy (liraglutide, exenatide) in controlled trial settings. In addition, the mechanisms of how GLP-1 induce hepatic insulin sensitivity have not been agreed. Earlier studies have attributed it to GLP-1's inhibitory effect on glucagon and subsequent decrease in hepatic glycogenolysis and gluconeogenesis (Toft-Nielson et al., 1996). In contrast, recent studies in rodents and humans have shown that the effect is likely independent of endogenous pancreatic hormones (Meneilly et al., 2001; Prigeon et al., 2003; Seghieri et al., 2013). Either by surgical removal of the pancreas in non-primate animal experiments (Sandhu et al., 1999) or by infusion of somatostatin analogues in humans (known to suppress secretion of pancreatic hormones) (Seghieri et al., 2013), these studies have found that the effect of GLP-1 on hepatic EGP is preserved even when endogenous glucagon and insulin secretion are suppressed (i.e. pancreatic clamp technique).

Excess influx of NEFA from adipose tissue (~59%) and hepatic DNL (~26%) are widely-recognised as the main sources of excess hepatic lipid accumulation in patients with NASH (Donnelly et al., 2005). Using the established  $^2\text{H}$  isotope tracer technique (Hazlehurst et al., 2013), our study is the first to report the impact of GLP-1 based therapy on DNL in patients

with NASH. Even though our reported basal percentage contribution of hepatic DNL to total hepatic lipid content is lower than previously reported (4.9%), we still observed a significant reduction in % hepatic DNL with liraglutide versus placebo (Figure 6.5). 5/7 patients on liraglutide successfully decreased hepatic DNL in the fasting-state after 12 weeks, whereas the remaining two had negligible differences. Of note, these two patients had the greatest reductions in fasting NEFA levels (-182, -219  $\mu\text{mol/L}$ ) on liraglutide, which might account for the lack of differences in DNL. Our findings are in keeping with mouse experiments of NAFLD, which have consistently reported anti-lipogenic changes in hepatic gene expression after chronic GLP-1 administration (Ding et al., 2006; Lee et al., 2007; Samson et al., 2008; Ben-Shlomo et al., 2011; Shirakawa et al., 2011; Svegliati-Baroni et al., 2011; Panjwani et al., 2013; Zhang et al., 2013). Most notably, these in-vivo studies have reported decreased hepatic gene expression of the rate-limiting enzymes involved in DNL (ACC1 and FAS) and the regulators of such as SREBP-1c and SCD1. Further study is, however, required to assess whether GLP-1 therapies have a direct effect on the rate of lipogenesis in hepatocytes, or whether GLP-1 induces its anti-lipogenic effect through the collective improvements seen in the metabolic phenotype.

There is a growing body evidence to suggest that adipose IR and subsequent excess release of NEFA into the circulation are linked with the onset of peripheral and hepatic IR (Kashyap et al., 2003; Boden, 2006), and progressive liver injury (Lomonaco et al., 2012). Therefore, reversal of such is likely to have significant implications on the progression of NASH and the associated cardiovascular risk profile. Our study highlights that liraglutide significantly reduces whole-body lipolysis in the fasting state (i.e.  $\downarrow$  serum NEFA,  $\downarrow$  Adipose IR index) and

enhances insulin-mediated suppression of lipolysis ( $\Downarrow$  INS- $\frac{1}{2}$ -max NEFA). Previous murine studies have reported similar effects on fasting NEFA levels with GLP-1 (Lee et al., 2007; Lee et al., 2012), but have not extrapolated on the actions of GLP-1 in the hyperinsulinaemic state. In support of our findings, Zander et al have shown that 6 weeks of continuous subcutaneous GLP-1 infusion in patients with type 2 diabetes (n=10) resulted in significant reductions in fasting and post-prandial serum NEFA (Zander et al., 2002). Similarly to our study and other murine models, these changes were accompanied by significant weight loss. Data from *in vitro* experiments, however, support the notion that GLP-1 may elicit some of these effects directly on adipocytes. Using 3T3 adipocyte cell lines, several authors have shown that GLP-1 increases insulin signaling by upregulation of phosphorylated IRS-1 and Akt (i.e. insulin signaling pathway), and in turn enhance glucose uptake (Egan et al., 1994; Wang et al., 1997; Gao et al., 2007; Vendrell et al., 2011). In vitro experiments, regarding the direct effect of GLP-1 on lipolysis are less consistent, and appear to be related to variations in dosage and culture conditions (Ruiz-Grande et al., 1992; Perea et al., 1997; Villanueva-Peñacarrillo et al., 2001; Sancho et al., 2005; Vendrell et al., 2011).

By directly measuring interstitial glycerol release (using in-situ microdialysis) we have demonstrated that liraglutide significantly decreases lipolysis and IR in abdominal SAT; a site previously shown to be of severe adipose dysfunction in patients with NASH (Chapter 5). Even though we did not measure adipose tissue blood flow as part of the microdialysis protocol, we are confident that these effects are attributed to insulin sensitivity (versus changes in insulin delivery), because data from previous studies have shown that GLP-1 does not effect the blood flow in SAT (Bertin et al., 2001). By sensitizing the abdominal SAT to



insulin, it is likely that liraglutide has increased the buffering capacity of SAT in response to external insults, most notably excess caloric intake. As a result, the liver and other metabolically active organs are likely to be rescued from the overspill of lipotoxic metabolites into the systemic circulation. Furthermore, we have shown that liraglutide reduces circulating levels of CCL-2 (a cytokine that attracts monocytes to disease tissue). This together with decreases in leptin may infer a reduction in adipose inflammation. In support of this theory, Shirakawa and colleagues have shown that DPP-4 inhibition (and therefore increased GLP-1 levels) prevents adipose infiltration of macrophages in a diabetes-induced mouse model (Shirakawa et al., 2011). Ideally this requires validation in humans by means of adipose tissue biopsy, which due to other tests taking precedent (i.e. liver biopsy) was not performed in our study. Furthermore in our study, liraglutide increased circulating adiponectin (anti-inflammatory, insulin sensitiser) in conjunction with decreased leptin (pro-inflammatory). The resultant reduction of 50% in leptin-to-adiponectin ratio was comparable to levels previously seen in our healthy volunteers (i.e. 1.55 vs. 1.32). In relation to this finding, liraglutide has recently been shown to increase the expression and secretion of adiponectin (Acrp30) from adipose tissue, adjacent to reversing liver injury in adiponectin knockdown mice (Zhang et al., 2013). Furthermore, increases in circulating adiponectin (either via direct replacement or secondary to therapies) have previously been implicated in the resolution of lipotoxic liver injury and fibrosis in humans (Xu et al., 2003; Gastaldello et al., 2010). Even though we can only speculate on the order of events in our NASH patients, it is likely that the positive effects of liraglutide on adipose tissue metabolism and lipolysis play an assisting role to the improvements we observed in hepatic insulin sensitivity and DNL with liraglutide.

One of main questions that remain is whether the anti-lipogenic and insulin-sensitising actions of GLP-1 in patients with NASH are independent of weight loss. A culmination of the sample size and the fact that all seven patients who received liraglutide lost weight (median 6 kg, range -4.5 to 7.6 kg) in our study, meant we were unable address this question. Rodent models of DPP-4 inhibition (Shirakawa et al., 2011) or genetic deficiency (Ben-Shlomo et al., 2011), in which circulating levels of endogenous native GLP-1 are elevated, have reported improvements in hepatic lipid accumulation in the absence of weight loss. Trevaskis et al confirmed these findings in ALIOS-fed mice on the GLP-1R agonist, exendin-4, using a pair-feeding approach to ensure weight equality between the treated and control animals (Trevaskis et al., 2012). Understanding the weight independent actions of GLP-1 on tissue-specific insulin sensitivity is more complicated. In contrast to studies in which weight differences were noted with GLP-1 therapy (Lee et al., 2007; Parlevliet et al., 2009; Zhang et al., 2013), Ben-Sholmo et al found no effect on either hepatic or peripheral IR in the weight-neutral DPP-4<sup>-/-</sup> rat model (Ben-Shlomo et al., 2011). In contrast, Gedulin and colleagues found greater than a 60% improvement in insulin sensitivity in diabetic obese Zucker rats treated with exendin-4 versus pair-fed controls, which were well matched for other metabolic parameters (including weight change) after 6 weeks of treatment (Gedulin et al., 2005). Whether these discrepancies were due to differences in the action of GLP-1 at physiological concentration (i.e. DPP-4 inhibition) compared to supra-physiological concentrations (i.e. liraglutide or exendin-4) remains unclear.

In summary, our prospective placebo-controlled study highlights that 12 weeks treatment with liraglutide significantly reduces adipose tissue insulin sensitivity, whole-body (and

localised SAT) lipolysis and adipose tissue inflammation. In parallel with changes in adipose metabolism, we observed significant improvements in hepatic insulin sensitivity and DNL. Collectively, these actions likely explain the metabolic improvements we observed in total/LDL cholesterol, fasting/chronic glycaemic control and liver enzymes. Whether liraglutide has a direct effect on adipose and liver tissue, independent of weight loss, requires further study. It is therefore possible that GLP-1 analogue therapy may represent a novel treatment for patients with NASH, although the safety and histological efficacy await the completion of the 48-week LEAN trial in 2014.

## CHAPTER 7: DIRECT EFFECT OF GLP-1 ON THE LIVER *IN-VITRO*

### 7.1 Introduction

Our studies to date have highlighted that GLP-1 based therapies, and in particular liraglutide, have unique pharmaceutical advantages in patients with NASH. Unlike other anti-diabetic drugs, which have a tendency to promote weight gain (i.e. TZDs), GLP-1 based therapies induce weight loss in addition to improving glucose homeostasis. Furthermore, by means of prospective controlled study we have found that liraglutide decreases liver enzymes, cholesterol, hepatic DNL, and most notably, insulin resistance in the liver and adipose (abdominal) tissue. It remains unclear, however, if these metabolic benefits are primarily due to improvements in glycaemic control and weight loss, or are a direct effect of GLP-1 signalling in the liver. Furthermore, a better understanding of whether the GLP-1R is present in the liver would enhance our knowledge of how GLP-1 exerts its anti-steatotic actions.

The GLP-1R is a transmembrane G-protein coupled receptor that is expressed in a variety of human tissues, including the pancreas (especially  $\beta$ -islet cells), lungs, and the central nervous system (Baggio and Drucker, 2007; Holst, 2007). However, it remains controversial as to whether the GLP-1R is expressed in liver tissue and more specifically, hepatocytes (Blackmore et al., 1991; Valverde et al., 1994; Wei and Mojsov, 1995; Bullock et al., 1996; Dunphy et al., 1998; Flock et al., 2007; Aviv et al., 2009; Liu et al., 2010; Tomas et al., 2011a). The majority of these studies have used either human hepatoma cell lines (Huh7, HepG2) or whole liver tissue/hepatocytes extracted from rodents. This together with the wide variety

of techniques utilised to investigate GLP-1R expression and differences in cell culture (Table 1.3; chapter 1), likely explain the inconsistencies in the field. Furthermore, there is a noticeable paucity of data in primary human liver tissue. This is particularly pertinent, as studies in thyroid and lung tissue have reported marked differences in GLP-1R expression between rodent and human species (Körner et al., 2007; Bjerre Knudsen et al., 2010).

The anti-steatotic effects of GLP-1 based therapies have consistently been reported in a variety animal models of NAFLD (diet induced +/- genetic manipulation), by means of oil red O staining and triglyceride quantification of liver tissue extracts (Ding et al., 2006; Samson et al., 2008; Parlevliet et al., 2009; Ben-Shlomo et al., 2011; Samson et al., 2011; Sharma et al., 2011; Shirakawa et al., 2011; Tomas et al., 2011a; Lee et al., 2012; Mells et al., 2012; Trevaskis et al., 2012; Panjwani et al., 2013). The majority of these studies have shown changes in hepatic gene±protein expression that indicate that the GLP-1's anti-steatotic is likely attributed to a combination of reduced hepatic DNL and increased  $\beta$ -oxidation; and to a lesser extent NEFA uptake and enhanced VLDL secretion. These anti-steatotic effects of GLP-1 were accompanied by significant weight loss in the majority of these studies, which is in keeping with our retrospective study (Armstrong et al., 2013d) and our recent observations in patients with NASH (Chapter 6). However, a few studies that have used models of DPP-4 inhibition ((Ben-Shlomo et al., 2011) or pair-feeding techniques (Trevaskis et al., 2012) to eliminate the effects of changes in weight, have reported similar improvements in hepatic lipid handling with exogenous or endogenous native GLP-1 peptides. These findings, however, require validation with *in vitro* experiments in order to completely eliminate any metabolic confounders.

Therefore, the aims of our *in vitro* studies were to: a) determine if primary human liver tissue and its cellular components (biliary epithelium, sinusoid endothelium, hepatocytes) express the pancreatic-type GLP-1R; b) determine if GLP-1 directly elicits changes on intracellular signaling and lipid accumulation; and c) use isotope tracer experiments to examine which mechanisms contribute to GLP-1's effect on lipid handling in the absence of metabolic confounders.

## **7.2 Methods**

### **7.2.1 Presence of GLP-1 receptor on liver cell types**

The presence of the GLP-1R in whole liver tissue and various liver cell types was examined using PCR and western blotting techniques. Due to the well-reported expression of GLP-1R in human pancreatic beta-islets (Baggio and Drucker, 2007), whole pancreas was used as a positive control throughout.

#### *7.2.1.1 Human liver sample collection*

All human liver tissue was obtained from fully informed and consented patients at the Queen Elizabeth University Hospital in Birmingham under local Research Ethical Committee approval (CA/5192). Explanted liver tissue and donor liver tissue was used within 12 and 24 hours of retrieval, respectively.

Whole liver tissue samples were resected from fresh, non-diseased human liver tissue from donor organs (n=10) that were surplus to transplant requirements (i.e. variable donor age and cold ischaemia time) and from NASH cirrhotic liver tissue (n=6) removed at transplantation. 30-50 mg samples were immediately placed in RNA later to enable RNA extraction or snap frozen with liquid nitrogen to be stored at -80 °C for future protein extraction. Human pancreas was sampled from normal margins of partial pancreatectomy (n=4) to serve as a positive control and stored in keeping with the Human Tissue Act.

#### *7.2.1.2 Primary Liver Cell isolation*

Primary biliary epithelial cells (BEC) (Joplin et al., 1989; Humphreys et al., 2010), human sinusoidal endothelial cells (HSEC) (Lalor et al., 2002) and human hepatocytes ((Bhogal et al., 2011) were isolated as per established protocols from a variety of cirrhotic liver disease tissue (including PSC, PBC) removed at transplantation and from non-diseased liver tissue from liver resection or donor organs that were surplus to transplant requirements. BEC (n=3), HSEC (n=3) and primary hepatocytes (n=3; with the guidance of Bhogal R) were cultured to confluence in their respective media (Joplin et al., 1989; Humphreys et al., 2010; Bhogal et al., 2011) on tissue culture flasks coated with rat tail collagen I, in a humidified atmosphere at 37°C in 5% CO<sub>2</sub> (Table 7-1). BEC and HSEC were used between passage 2 and 5 to ensure phenotypic stability, whereas primary hepatocytes were used within 48 hours of isolation and only if cell viability >80%.

#### *7.2.1.3 Liver-derived hepatoma Cell Lines (Huh 7) culture*

Huh 7 cells (American Type Culture Collection) were cultured on non-coated tissue culture plates/flasks in Dulbecco's Modification of Eagle's Medium (DMEM), supplemented with 10% v/v heat inactivated foetal calf serum (FCS) (Gibco®), 1% nonessential amino acids (Invitrogen), 2 mM glutamine (Invitrogen), 10,000 units/l Penicillin and 10 mg/ml Streptomycin (Invitrogen). Cells were cultured in humidified atmosphere at 37°C in 5% CO<sub>2</sub>. For all experiments, cell viability and density was determined by 0.4% Trypan Blue (1:1 dilution) exclusion counting with a hemocytometer.



Isolate Primary Cell type	Primary Media	Media supplements				
		Serum	Antibiotics	Growth factors	Amino acids	Additional
BEC	DMEM (4.5g/L D- glucose; Gibco)	10% human serum (HD supplies)	Penicillin (100U/ml Gibco) Streptomycin (100µg/ml; Gibco)	EGF (10ng/ml; Peprotech) HGF (10ng/ml; Peprotech) Triiodothyronine (2nM; Sigma) Insulin (0.124 U/ml; Sigma)	L-glutamine (2nM; Gibco)	Hydrocortisone (2µg/ml, UHB Birmingham) Cholera toxin (10ng/ml; Sigma)
HSEC	EBM (invitrogen)	10% human serum (HD supplies)	Penicillin (100U/ml Gibco) Streptomycin (100µg/ml; Gibco)	VEGF (10ng/ml; Pep) HGF (10ng/ml; Peprotech)	L-glutamine (2nM; Gibco)	
Hepatocytes	Williams E (arginine- free; Sigma)	-	Penicillin (100U/ml Gibco) Streptomycin (100µg/ml; Gibco)	Insulin (0.124 U/ml; Sigma)	L-glutamine (2nM; Gibco) Ornithine (0.4 mmol/l; Gibco).	Hydrocortisone (2 ug/ml)

**Table 7-1. Culture media for different liver cells types.**

Key: BEC, Biliary epithelial cell; DMEM, Dulbecco's Modification of Eagle's Medium; EBM, endothelial basal media; EGF, epidermal growth factor; HGF, hepatocyte growth factor HSEC, human sinusoidal endothelial cell; VEGF, vascular endothelial growth factor.

#### 7.2.1.4 RNA extraction

Total RNA was extracted with the Qiagen RNeasy kit (Qiagen) according to the manufacturer's protocol for human tissue (30mg samples) and cells in culture (confluent T25 flasks [all]; confluent 12-well for primary hepatocytes). Prior to RNA extraction, all primary cell types were starved of serum, insulin, growth factors and additional supplements for 4 hours in their respective medias, in keeping with previous reports (Gupta et al., 2010). In the case of Huh 7 cell lines, serum starvation was performed for 12 hours (Gupta et al., 2010).

Human and foetal calf serums were replaced with 0.5% bovine serum albumin (BSA; Sigma) for serum starvation. Cells were washed in cold phosphate buffer saline (PBS) three times and underwent direct cell lysis with 350 $\mu$ L of RLT buffer (with 1:100  $\beta$ -mercaptoethanol). The cell suspension was vortexed in micro-centrifuge tubes and passed through an 18 gauge needle five times to shear the DNA. 350 $\mu$ L 70% ethanol was added to each tube and the total 700 $\mu$ L of cell suspension was pipetted into the RNeasy mini-spin column with a 2ml collection tube. NanoDrop (Thermo Scientific) was used to measure purity (ratio of absorbance) and quantity (ng/ $\mu$ L) of the RNA extracted. RNA was only used if the ratios of absorbance (260/280; 260/230) were between 1.80-2.10 and concentration was >100ng/ $\mu$ L on the nanodrop.

#### *7.2.1.5 Reverse transcription to cDNA*

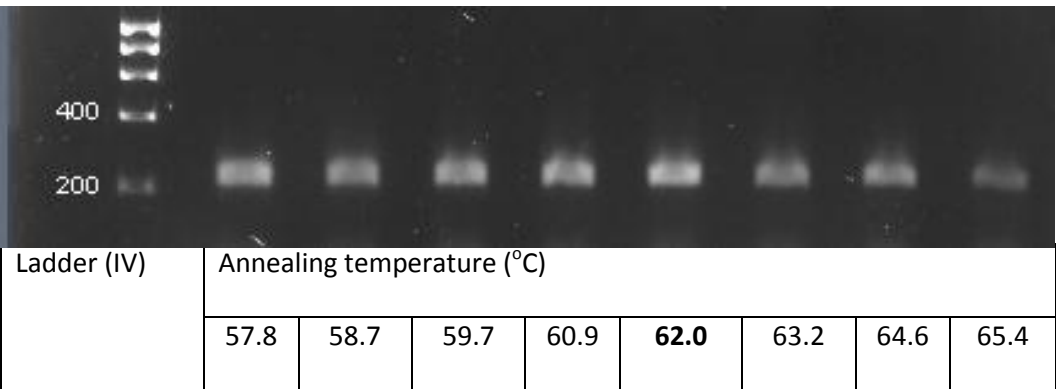
Reverse transcription (RT) was carried out using 1 $\mu$ g of extracted RNA with a standard protocol (using random primers [Promega], SuperScript<sup>TM</sup> II Reverse Transcriptase [Invitrogen]). RT mix was incubated at room temperature for 10 min, followed by 50 mins at 42°C and 15 mins at 70°C. Thereafter, cDNA was stored at -20 °C.

#### *7.2.1.6 Non-quantitative PCR of GLP-1R expression*

1 $\mu$ L of each cDNA sample was mixed with 8 $\mu$ L Biomix Red (Bioline) reaction mix containing Taq DNA polymerase and 0.5 $\mu$ L of each forward and reverse GLP-1R primers (Sigma).

Primer-BLAST was used to identify suitable primers that were GLP-1R specific (Ye et al., 2012) and that spanned a minimum of three introns to avoid amplifying any contaminating genomic DNA. Subsequently, the following primers were used for GLP-1R; *forward* 5'-GTTCCCCTGCTGTTTGTGT-3'; *reverse* 5'-CTTGGCAAGTCTGCATTGA.

Samples (reaction mix 10µL) were held at 4°C before PCR amplification with a G-storm thermal cycler. The following cycling program was used: 5 min denaturation at 95°C, followed by 35 cycles of 45 seconds at 95°C, 1 minute at 62°C annealing temperature and 45 seconds at 72°C with a final extension for 10 minutes at 72 °C. Samples were held at 4°C overnight. Of note, the annealing temperature of the GLP-1R primers was optimized using whole pancreas cDNA (Figure 7-1). After PCR amplification, samples (10µL per lane) were electrophoresed in 2% agarose gel and stained with ethidium bromide. Whole pancreas cDNA was used as a positive control and RT negative samples used as negative controls. 18S (Sigma) was used as the 'house keeping gene' throughout.



**Figure 7-1. Optimisation of annealing temperature for GLP-1R primers**

Whole human pancreas used as a positive control. 35 cycles performed. Temperature gradient from 57.8-65.4°C, with an optimal annealing temperature of 62°C (**bold**).

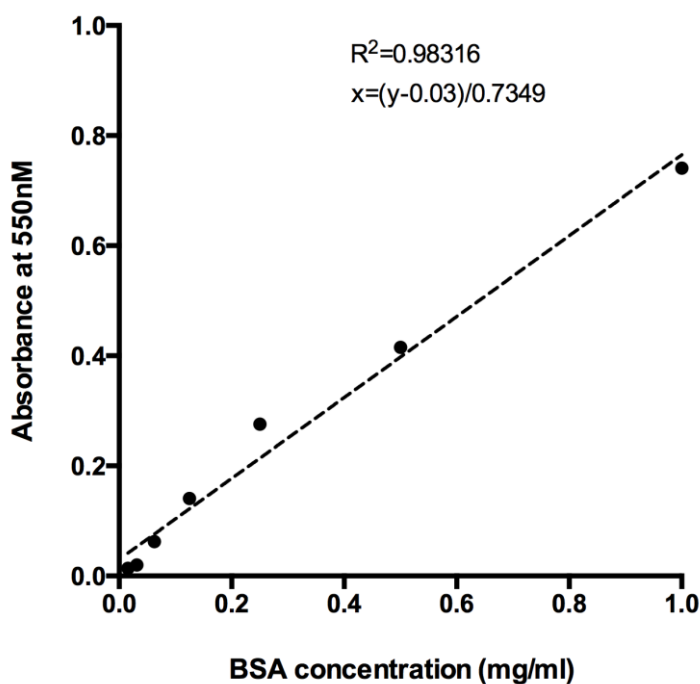
PCR was carried out for the presence of GLP-1R in whole donor liver (n=5), BEC (n=5), HSEC (n=4), HUH 7 (n=4) and primary hepatocytes from explanted livers (n=4).

#### *7.2.1.7 Quantitative real-time PCR (qPCR) of GLP-1R expression*

Expression of the GLP-1 receptor gene was quantified by qPCR relative to the house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). QuantiTect primers (GLP1R: QT00066780; GAPDH: QT01192646) and the QuantiFast SYBR green Taq-man polymerase real-time PCR kit (all from Qiagen) were used, as described previously (Amisten, 2012). The size of each qPCR product was determined using agarose gel electrophoresis and compared to the amplicon size (119bp for GAPDH and 130bp for GLP-1R) stated by the manufacturer of the QuantiTect primers (Qiagen). Genes were quantified if their expression level were within the linear range of amplification, as determined by performing quantifications using the same primers and serially diluted cDNA templates. Genes that were detected outside the linear range of quantification were assigned as present at trace levels only, as they could not be quantified using the above primers and qPCR kits. In addition, to the samples used for non-quantitative PCR, a further 7 samples of whole donor liver were analysed (n=10 on total). qPCR was performed in collaboration with Dr Stefan Amisten (Oxford University).

#### 7.2.1.8 Western blotting for GLP-1R expression

Protein lysates were prepared from Huh7 cell lines, primary hepatocytes, donor human liver and human pancreas using CellLytic MT Buffer mixed with protease inhibitor cocktail and DNase-1 (Sigma), at 1:100 dilutions. The protein concentration of each sample was determined using the Micro Lowry, Onishi and Barr Modification Protein Kit (Sigma), in accordance with the manufacturer's instructions. BSA was used as the standard (Figure 7-2).



**Figure 7-2. Standard curve for protein quantification**

Serial dilutions of BSA were made from a starting concentration 1mg/ml. A Linear response of absorbance versus protein was observed.

Samples (40  $\mu$ g protein) were resolved on a 12% bis-acrylamide gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose membrane (Amersham Pharmacia Biotech). Membranes were blocked for 1 hour at room temperature

in PBS/0.1% Tween-20. The membrane was subjected to immunodetection with primary antibodies for GLP-1R. The following polyclonal anti-rabbit antibodies against GLP-1R were tested: AB39072 (Abcam), SC-66911 (Santa Cruz Biotechnology) and LS-A1206 (Lifespan Biosciences), at dilutions ranging from 1:100 to 1:500. The primary antibodies incubations were carried out overnight at 4°C in PBS/0.1% Tween-20 containing 5% w/v non-fat dried milk. Antibodies were detected using horseradish peroxidase conjugated rabbit anti-mouse IgG (1:1000-1:2000 dilution; DAKO) for 1 hour at room temperature. Protein bands were visualised using enhanced chemiluminescence (ECL) (Pierce Perbio) or West PICO Chemiluminescent Substrate SuperSignal (Thermo Scientific Pierce) and exposure to Hyperfilm-ECL (Amersham Pharmacia Biotech). To ensure that equal amounts of protein were loaded and transferred onto membranes, the membranes were stripped and re-probed for the anti-mouse monoclonal antibody to beta-actin (1:2000; Sigma) and stained with Ponceau S solution (Sigma).

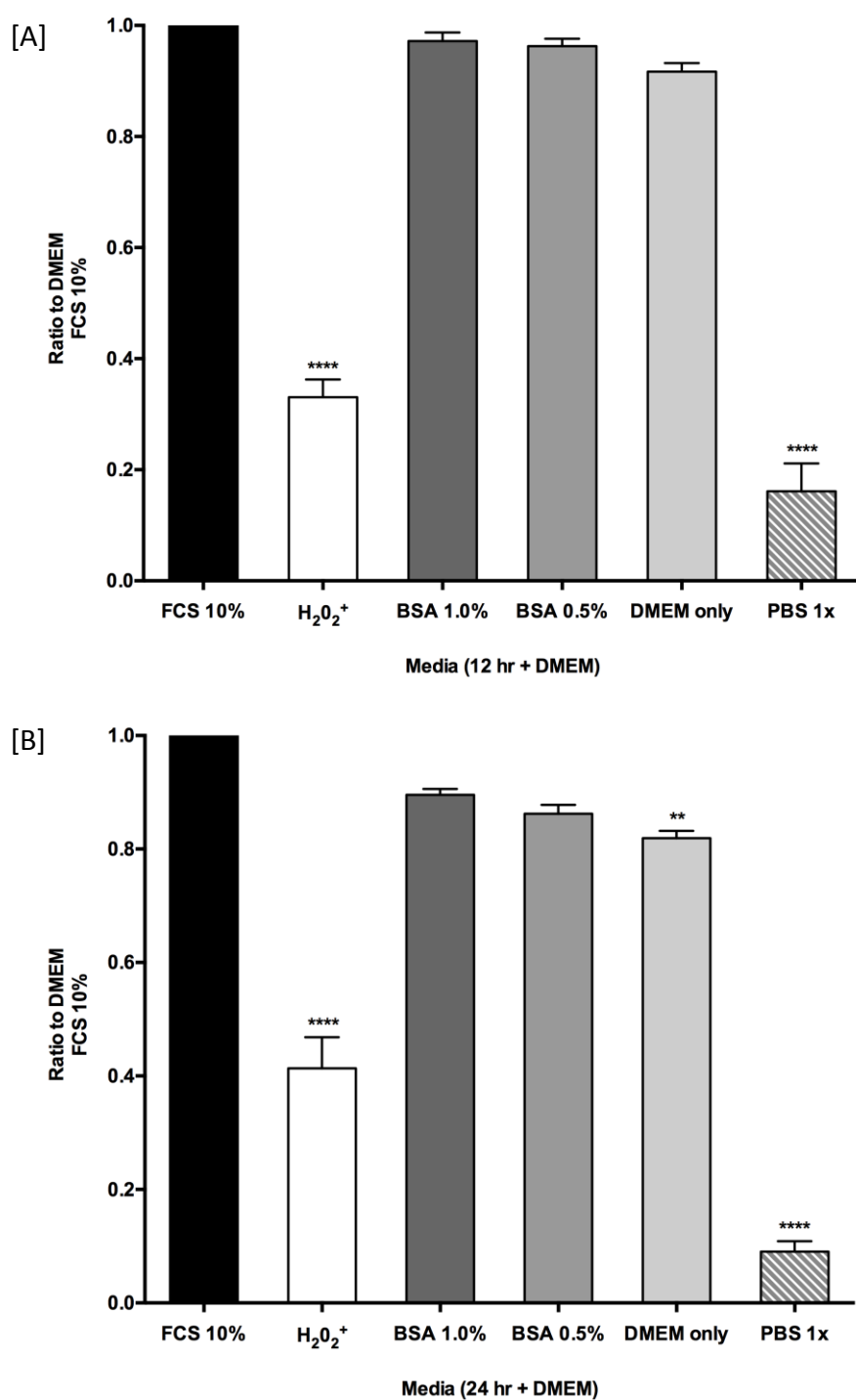
## **7.2.2 Direct effect of GLP-1 analogues in human hepatocytes**

### *7.2.2.1 Effect of GLP-1 treatment on hepatocyte cell viability*

Principle: Prior to performing functional experiments the effects of different culture media (serum-free) and GLP-1 treatment on Huh 7 cell viability were assessed using a methylthiazolyldiphenyl-tetrazolium (MTT) bromide assay (Invitrogen). The MTT assay detects the ability of mitochondrial enzymes and specifically succinate dehydrogenase to metabolise MTT.

Method: Huh 7 cells were seeded on non-coated flat-bottom 96-well plates at a density of  $12 \times 10^3$  cells per  $32 \text{ mm}^2$  surface area and allowed to reach 90% confluence. To optimize serum-free culture media conditions, Huh 7 cells were cultured in DMEM FCS 10% (*control*), BSA 1.0%, BSA 0.5%, and DMEM only for 12 hrs and 24 hours (Figure 7-3). Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) at 1mmol/L (2hr only) and PBS (x1) were used as positive controls.

After media optimization, Huh 7 cells were treated with GLP-1 7-37 (1nM, 10nM, 100nM), Exendin-4 (1nM, 10nM, 100nM) and Exendin Fragment 9-39 (1  $\mu\text{M}$  (Sigma); GLP-1R inhibitor) in DMEM BSA 0.5% for 12 hours. For the last 4 hours of the 12 or 24 hr incubation, 10  $\mu\text{l}$  of MTT solution (5 mg/ml; Sigma) was added to 100  $\mu\text{l}$  of the relevant medium at  $37^\circ\text{C}$ . The 96-well plates were then centrifuged at 200g for 10 minutes and the supernatant removed to leave dry formazan crystals in the wells. The crystals were dissolved using 100  $\mu\text{l}$  of a 1:1 mixture of dimethyl sulfoxide:100% ethanol and placed on an orbital microplate shaker for 10 minutes at 500 rpm. Optical density was read immediately at 490 nm. Experiments were carried out three times with each condition in triplicate. Results were expressed as ratios of DMEM FCS 10% in the serum-free media experiments and DMEM BSA 0.5% in the GLP-1 experiments.



**Figure 7-3. Huh-7 cell viability in various medias**

MTT assay performed to assess optimal conditions for 12 hr [A] and 24 hr [B] serum-starvation. <sup>+</sup>H<sub>2</sub>O<sub>2</sub> (1 mmol/L for 2hr only) and PBS (x1) used as positive controls. \*\*p<0.01, \*\*\*\*p<0.0001.



#### 7.2.2.2 Effect of GLP-1 on G-protein coupled receptor signalling in hepatocytes

Principle: The cAMP assay was used to determine if GLP-1 interacts directly with hepatocytes via a G-protein coupled receptor (GPCR) (Beavo and Brunton, 2002). cAMP is an important second messenger in many metabolic *intracellular* signal transduction pathways. Binding of extracellular stimuli (i.e. glucagon, adrenaline) to GPCRs activates the enzyme, adenylyl cyclase, which is located on the inner membrane. In turn, it converts ATP to cAMP, thereby increasing the intracellular levels (Kamenetsky et al., 2006).

Method: Huh 7 cells were seeded on non-coated tissue culture plates at a density of  $5 \times 10^5$  cells per  $950 \text{ mm}^2$  surface area (i.e. per 6-well). Cells were propagated to 90% confluence in DMEM with 10% FCS. Prior to treatment, cells were washed with PBS (1X) three times and underwent serum starvation for 12 hours in DMEM supplemented with BSA 0.5%, at which the cell density was  $1 \times 10^6$  cells per  $950 \text{ mm}^2$  surface area. To determine whether GLP-1 receptor agonists could promote a sustained increase in hepatocyte cAMP production, cells were treated with either Exendin-4 (10 nM or 100 nM) or active GLP-1 7-37 (10nM or 100nM) in DMEM with 0.5% BSA for 3 hours. DMEM with BSA 0.5% and Forskolin (10  $\mu\text{M}$ ; R&D systems) served as negative and positive controls, respectively. Repeat experiments were undertaken using Exendin Fragment 9-39 (1  $\mu\text{M}$ ; Sigma), a competitive antagonist for GLP-1 or Exendin-4 receptor binding, to assess whether cAMP production was directly through GLP-1R activation (i.e. GPCR). Exendin 9-39 (1  $\mu\text{M}$ ) was used for 30 minutes followed by GLP-1, Exendin-4 or Forskolin for 3 hours.

cAMP measurements were carried out on whole cell lysates using a competitive cAMP ELISA kit (R&D systems). To prepare whole cell lysates, cells were washed three times in cold PBS and then 300  $\mu$ L cell lysis buffer was applied to each well of  $1 \times 10^6$  cells. Cells underwent a freeze ( $-20^{\circ}\text{C}$ ) thaw cycle twice and then cellular debris was removed via centrifugation at 600g for 10 minutes at  $4^{\circ}\text{C}$ . The supernatant was assayed immediately as per manufacturer's instructions (R&D systems). The optical density of each well was determined using a microplate reader set to 450 nm (with wavelength correction set to 540 nm). Duplicate readings were averaged and the average non-specific binding optical density was subtracted. For each experiment a standard curve was created using GraphPad 6.0 Prism software capable of generating a four parameter logistic curve-fit. A linear response of absorbance versus cAMP standards was observed in all experiments ( $R^2 > 0.990$ ; in-house coefficient of variation of 2.9-7.1%) Experiments were carried out three times in triplicate and results were expressed in pmol/L. $10^6$  cells.

#### *7.2.2.3 Effect of GLP-1 treatment on hepatocyte lipid metabolism*

For all lipid metabolism experiments Huh 7 cells were seeded on non-coated tissue culture plates at a density of  $1 \times 10^5$  cells per  $190 \text{ mm}^2$  surface area (i.e. per well of 24-well plate). The only exception was the triglyceride quantification assay in which 6-well plates were used ( $1 \times 10^6$  cell per well prior to treatment). Cells were propagated to 80% confluence in DMEM with 10% FCS. Prior to treatment, cells were washed with ice cold PBS (1X) three times and underwent serum starvation for 12 hours (overnight) in DMEM supplemented with 0.5% NEFA-free BSA (Sigma). Prior to commencing treatment there were approximately  $1.8 \times 10^5$

cells per well. For each experiment DMEM with 0.5% BSA (i.e. serum-free media) and insulin (5nM) served as negative and positive controls, respectively. Low-glucose (1.0g, 3mM glucose) DMEM was used, instead of the standard 4.5 g glucose DMEM, for the fatty acid uptake and beta-oxidation experiments to mimic the fasted state without inducing cell death.

#### 7.2.2.3.1 ANTI-STEATOTIC EFFECT OF GLP-1 ON HEPATOCYTES

Principle: The most abundant NEFA present in the human diet and in serum, namely palmitic acid (a saturated NEFA) and oleic acid (a mono-unsaturated NEFA) have been previously shown to increase intracellular triglyceride accumulation when added to the growth media of hepatocytes (Ricchi et al., 2009; Gupta et al., 2010). Studies have shown oleic acid to be less toxic than palmitic acid and to attenuate palmitic acid-induced toxicity in steatosis models *in vitro* (Ricchi et al., 2009). Therefore, following NEFA loading, the effect of GLP-1 on triglyceride accumulation in hepatocytes was determined with Oil Red O staining and quantified using a commercial triglyceride assay (Biovision).

Methods: Huh7 cells were exposed to DMEM containing 0.5% FFA-free BSA and NEFA-loaded with a mixture of 200  $\mu$ M palmitic and 200  $\mu$ M oleic acid (Sigma). This concentration of NEFAs was used, as at 400  $\mu$ M each the cells had poor viability, with <50% surviving the duration of the experiments. After 12 hours, cells were carefully washed three times with PBS (x1) treated with controls, exendin-4 10nM and 100nM for a further 12 hours. After a

further three washes with PBS (x1), cells were either fixed for Oil Red O staining or lysed for triglyceride quantification.

*Oil red O Staining:* Cells were fixed with formalin for 5 mins, washed with PBS (x1) and then incubated with 450  $\mu$ L 60% isopropanol (Sigma) for 5 mins. 450  $\mu$ L Oil Red O working solution was then added to each well and incubated for 45 minutes with gentle rocker. Of note, Oil Red O working solution was made by dissolving and incubating 0.25g of Oil Red O powder (Sigma) in 50 ml 100% isopropanol overnight at 37°C, followed by filtering the excess solute off. After removal of the oil red solution, a further 450  $\mu$ L of 60% isopropanol was added and left for 5 mins. After which the cells were washed three times with 450  $\mu$ L of deionised water and visualised using a 40X microscope. Experiments were carried out three times in triplicate.

*Triglyceride quantification assay:* The assay was performed in keeping with the manufacturers instructions (Biovision #K622-100). In brief, Huh 7 cells were lysed in 1ml 5% NP-40 lysis buffer, placed in an eppendorph and heated to 80°C in a water bath until the solution went cloudy. The cell suspension was then cooled to room temperature and then re-heated to ensure the triglyceride had solubilised. The sample was then centrifuged at 14000 rpm (2 min) to remove insoluble material and then diluted 5-fold with sterile deionised water. Of note, 10-fold dilution was attempted but resulted in undetectable levels of triglyceride in the non-NEFA control. A Standard curve was prepared by diluting 2mM triglyceride standard with the assay buffer provided to generate 50  $\mu$ L of standards at concentrations of 0, 2, 4, 6, 8 and 10nM/well. 2 $\mu$ L of lipase was then incubated at room

temperature for 20 min with 50  $\mu$ L of either sample or standard in a 96-well plate to convert triglyceride to glycerol and NEFA. 50  $\mu$ L of triglyceride reaction mix (46  $\mu$ L assay buffer, 2  $\mu$ L triglyceride probe, 2  $\mu$ L triglyceride enzyme mix) was added to each well and incubated for 60 mins in the dark. The optical density of each well was determined using a microplate reader set to 570 nm. A linear response of absorbance versus triglyceride standards was observed ( $R^2=0.950$ ). Experiments were carried out three times in triplicate and results were expressed in nmol/L per  $10^6$  cells.

#### 7.2.2.3.2 EFFECT OF GLP-1 ON HEPATIC *DNL* IN HEPATOCYTES

Principle: DNL is a key component of lipid accumulation within the liver (Donnelly et al., 2005). DNL encompasses fatty acid synthesis and subsequent triglyceride synthesis (when NEFA are esterified with glycerol to form fats). A key step of fatty acid synthesis is the conversion of acetyl CoA to malonyl CoA in the cytoplasm of the hepatocytes, and its subsequent conversion to fatty acid (Wakil et al., 1983). This key reaction is catalysed by the enzyme ACC1, which itself is de-phosphorylated and activated by insulin. This assay measures the incorporation of a 1- $[^{14}\text{C}]$ -labelled acetic acid tracer combined with unlabelled ('cold') sodium acetate in cells (Jamdar, 1978), which have been treated with either insulin and/or GLP-1 analogues. After incubation cellular lipids are extracted and the retained  $^{14}\text{C}$  radioactivity is measured by scintillation counting.

Method for Huh 7: DNL was measured by the amount of uptake of 1- $[^{14}\text{C}]$ -acetate into the lipid component of hepatocytes, as described previously (Jamdar, 1978; Hazlehurst et al.,

2013). After culture in serum-free media, cells were incubated for 12 hours in 500  $\mu$ l of serum free media containing Exendin-4 (10 nM or 100 nM). 1-[ $^{14}$ C]-acetic acid [0.12  $\mu$ Ci/L; Perkin Elmer) with unlabelled sodium acetate [10  $\mu$ M] was added to the treated serum-free media for an additional 6 hours (= total 18 hours incubation). The incubation periods were optimized specifically for Huh 7 cell-lines (i.e. repeated experiments with 1 and 6 hour incubations prior to the addition of the isotopes – a total of 7 and 12 hours incubation, respectively). After incubation at 37  $^{\circ}$ C, cells were washed three times with ice cold PBS (1X), scraped into 250  $\mu$ l PBS, and transferred into glass tubes. The media was discarded. To extract the lipid fraction, 5 ml Folch solvent (chloroform:methanol 2:1) was added to the cells and vortexed vigorously for 20 seconds, after which 1ml distilled water was added and vortexed for a further 1 min. The glass tubes were then centrifuged at 300g for 5 mins to separate the sample into two distinct phases - aqueous (upper layer) and solvent (lower layer) - with protein collecting at the interface. The aqueous layer was aspirated off and the solvent was transferred to a scintillation tube to evaporate until dryness using a sample dryer in a fume cupboard overnight. Once dry, 5 mls of cold scintillation cocktail (ScintiSafe 3, Perkin Elmer) was added to each tube and the  $^{14}$ C radioactivity retained in the cellular lipid was determined by scintillation counting, using the liquid scintillation analyzer 2500 RT/AB (Packard, A Canberra Company, Oxfordshire, UK). The  $^{14}$ C radioactivity retained in the cellular lipid was expressed as disintegrations per minute (dpm)/per well. Experiments were carried out four times in quadruplicate.

Method for primary hepatocytes: All DNL experiments were then repeated using cryopreserved primary human hepatocytes, which were purchased from Celsis IVT

Technologies (M00995-P; Baltimore, Maryland). These hepatocytes were selected over explanted isolated (*in-house*) hepatocytes due to the flexibility of availability and ability to select donor status (specifically low-metabolic risk profile) with the purchased cells. In all four cases, hepatocytes were from non-obese, non-diabetic male donors, with no history of liver disease or excess alcohol history (n=4, median age 59.0 yrs, BMI 28.4 Kg/m<sup>2</sup>; Table 7-2).

Donor characteristics	Donor (LOT No.)			
	QOQ	LTG	CPQ	FOP
Age (years)	66	56	31	62
Gender	Male	Male	Male	Male
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian
BMI (Kg/m <sup>2</sup> )	29.1	27.8	25.9	29.0
Viral Status (HBV/HCV/HIV/CMV)	Negative	Negative	Negative	Negative
Alcohol history (drinks/day)	2	0	0	0
Smoker	Yes	No	Chewed tobacco	No
Drug history	No	No	Yes*	No
Diabetes status	Negative	Negative	Negative	Negative
Cause of Death	CVA Lung Ca	CNS tumour	Accidental trauma	Subdural haematoma
<b>Hepatocyte characterisation (assay rates)</b>				
CYP1A2/2A6/2C9/2D6/2E1/3A4 ECOD/UGT (pmol/10 <sup>6</sup> cells/min)	>10.0	>10.0	>10.0	>10.0
<b>Post-thaw hepatocyte viability (<i>in-house</i>)</b>				
Cell viability (trypan blue exclusion)	94%	90%	88%	82%
Number of viable cells (per ml plating media)	56 x 10 <sup>4</sup>	48 x 10 <sup>4</sup>	68 x 10 <sup>4</sup>	36 x 10 <sup>4</sup>

**Table 7-2. Donor characteristics and cell viability of primary hepatocytes**

Cytochrome P450 (CYP) assays performed by Celcis prior/post cryopreservation. Key: BMI, body mass index; CMV, cytomegalovirus; CNS, central nervous system; ECOD, ethoxycoumarin O-deethylase; UGT, uridine diphosphate glucuronosyltransferases; HBV/HCV, hepatitis B/C virus; HIV, human immunodeficiency virus. \*marijuana/cocaine.

Plating and culture media were prepared by mixing 1ml of *Torpedo*<sup>TM</sup> antibiotic mix (Z990008; Celcis) with 45 ml of *InvitroGRO*<sup>TM</sup> CP medium (Celcis) or serum/supplement-free Williams' E medium (Sigma), respectively. Primary human hepatocytes were thawed instantly, placed in plating media with gently mixing and equally pipetted into collagen I-coated 24-well plates (BD Biosciences), as previously described (Diao et al., 2010). After 24 hours, hepatocytes were washed gently with PBS (X1) and serum-starved in Williams' E media for 4 hours. After, which cells were treated as described *above*.

#### 7.2.2.3.3 EFFECT OF GLP-1 ON HEPATOCYTE NEFA UPTAKE

Principle: NEFA that are required for energy homeostasis and triglyceride synthesis in the liver are available from the adipose-derived NEFA plasma pool and hepatic fatty acid synthesis/DNL (Donnelly et al., 2005). The plasma NEFA concentration are derived from lipolysis in adipocytes, which occurs mainly in the fasting state and is repressed by insulin (Tamura and Shimomura, 2005; Bechmann et al., 2012). NEFA are then taken up by hepatocytes in a facilitated fashion by specific binding/transport membrane proteins (i.e. fatty acid binding protein, fatty acid transport protein), and not by a passive process (Berk, 2008). This assay measures the intracellular (cytosolic) accumulation of a 9,10-[<sup>3</sup>H]-labelled palmitate tracer after treatment with either insulin and/or GLP-1 analogues. After incubation intracellular lipids are extracted and the retained <sup>3</sup>H radioactivity is measured by scintillation counting.



Methods: NEFA uptake was measured by intracellular accumulation of 9,10- $^3\text{H}$ -palmitate, as previously described (Gathercole et al., 2011). After culture in low-glucose serum-free media (1.0g glucose DMEM with 0.5% BSA), cells were incubated for 6 hours in 300  $\mu\text{l}$  of low-glucose serum free media containing Exendin-4 (10 nM or 100 nM). The cells were then incubated at 37  $^{\circ}\text{C}$  for a further 12 hours with 300  $\mu\text{l}$  low-glucose serum free media containing 0.12  $\mu\text{Ci/L}$  9,10- $^3\text{H}$ -palmitic acid (Perkin Elmer) with unlabelled 'cold' palmitate (total concentration = 100  $\mu\text{M}$ ) and treatment with exendin-4 (10 nM or 100 nM) with and without 5nM insulin. The formation of the radiolabelled palmitate mixture is summarized in Box 7.1. The total treatment incubation periods were optimized specifically for Huh 7 cells with repeated experiments, including 12 hours (no pre-treatment, 12 hours isotope + treatment), 18 hours (6 hours pre-treatment, 12 hours isotope + treatment) and 24 hours (no pre-treatment, 24 hours isotope + treatment).

After incubation at 37  $^{\circ}\text{C}$ , the media was removed and placed into ependorpha for the beta-oxidation assay (*see below*). Cells were washed three times with ice cold PBS (1X), scraped in 250  $\mu\text{l}$  1% triton, and the cell lysate was then transferred into scintillation vials. 4 ml of cold scintillation cocktail (ScintiSafe 3, Perkin Elmer) was added to each tube and shaken vigorously. The intracellular  $^3\text{H}$  radioactivity, which represents the amount of palmitate uptake, was determined by scintillation counting and expressed as dpm/per well.

**Box 7.1 – Formation of ‘hot’ and ‘cold’ palmitate in serum-free media**

1. 100mM ‘cold’ palmitate

- Heat 1 ml 99% methanol with 100mM NaOH on a hot plate (50°C) and then dissolve 0.02564g palmitic acid (>99%, Sigma)

2. Serum-free media with ‘hot’ and ‘cold’ palmitate stock solution (500μM palmitate)

- Pre-heat 12.835 ml serum-free low-glucose (1g) DMEM (with 0.5% fatty acid-free BSA) at 37 °C
- Add 65 μL 100mM ‘cold’ palmitate and 100 μL 1mCi/ml 1,9-[3H]-palmitate (Perkin Elmer) to the pre-heated media

3. ‘hot’ and ‘cold’ palmitate treatment solution (100 μM palmitate) for 24-well plate

- Pre-heat 5.925 ml serum-free low-glucose (1g) DMEM (with 0.5% fatty acid-free BSA) at 37 °C
- Add 1.5ml of ‘hot’ and ‘cold’ palmitate stock solution (step 2) and 75 μL 100mM L-carnithine (final concentration 1mM)
- Divide the resultant solution and add the specific treatments accordingly (i.e. Exendin-4)

7.2.2.3.4 EFFECT OF GLP-1 ON  $\beta$ -OXIDATION OF NEFA IN HEPATOCYTES

Principle: NEFA  $\beta$ -oxidation occurs in the mitochondria and facilitates the degradation of activated NEFA to acetyl-CoA. It is a rapid and effective metabolic pathway for the allocation of energy within the liver (especially in the fasted state). Prior to  $\beta$ -oxidation, NEFA are activated by acyl-CoA-synthetase to fatty acid acyl-CoA substrates in the cytosol to facilitate uptake into the mitochondria. Long-chain NEFA, such as palmitate, are transported across the mitochondrial membrane via CPT1 (McGarry and Brown, 1997). Once inside the mitochondria the enzyme, Acyl-CoA dehydrogenase, catalyses the breakdown of long-chain NEFA acyl-CoA into acetyl CoA molecules. This assay measures  $\beta$ -oxidation by quantification

of [ $^3\text{H}$ ]-labelled  $\text{H}_2\text{O}$  generated by acyl-CoA dehydrogenase. The [ $^3\text{H}$ ] radioactivity of  $\text{H}_2\text{O}$  generated and released by the cells into the media is measured by scintillation counting (Kler et al., 1992).

Method: The rate of  $\beta$ -oxidation was measured by the conversion of 9,10- $^3\text{H}$ -palmitate (Perkin Elmer) to [ $^3\text{H}$ ] labelled- $\text{H}_2\text{O}$ , using a modification of the method described by Gathercole et al (Gathercole et al., 2011). After incubation, the 250  $\mu\text{L}$  media recovered from the NEFA uptake experiment (*described above*) was precipitated with 600  $\mu\text{L}$  10% trichloroacetic acid to remove the excess 9,10- $^3\text{H}$  palmitate] and centrifuged at 10,000 rpm for 5 mins. An aliquot of the resultant supernatant (800  $\mu\text{L}$ ) was treated with 2.5ml of 2:1 methanol:chloroform solution and 1ml of 2M KCL:HCL, and centrifuged at 3000g for 5 minutes. The resultant aqueous supernatant (2ml) was placed into a scintillation vial and counted following the addition of 3ml scintillation fluid (ScintiSafe 3, Perkin Elmer). The  $^3\text{H}$  radioactivity released into the media, which represents the rate of  $\beta$ -oxidation, was expressed as dpm/per well.

### **7.2.3 Statistical Analysis**

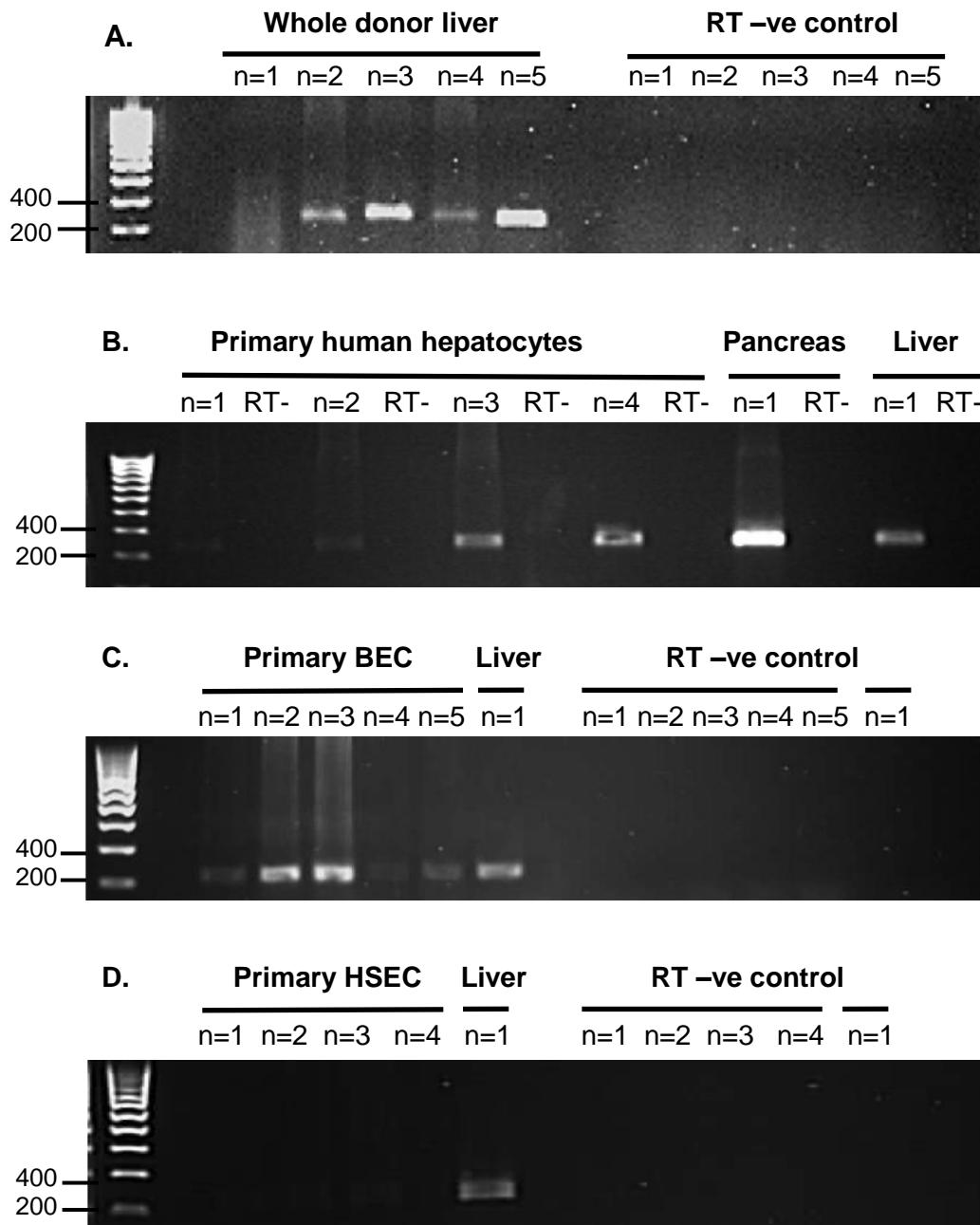
The data are presented as the mean  $\pm$  SE, unless otherwise stated. For comparison of two-treatment arms, paired t tests have been used (or nonparametric equivalents in which data were not normally distributed). ANOVA with Dunnett's post hoc analysis was used for comparisons of multiple doses and/or treatments. Statistical analysis was performed using Graphpad PRISM version 6.0 software ([www.graphpad.com](http://www.graphpad.com)).

## 7.3 Results

### 7.3.1 GLP-1R expression on liver cell types

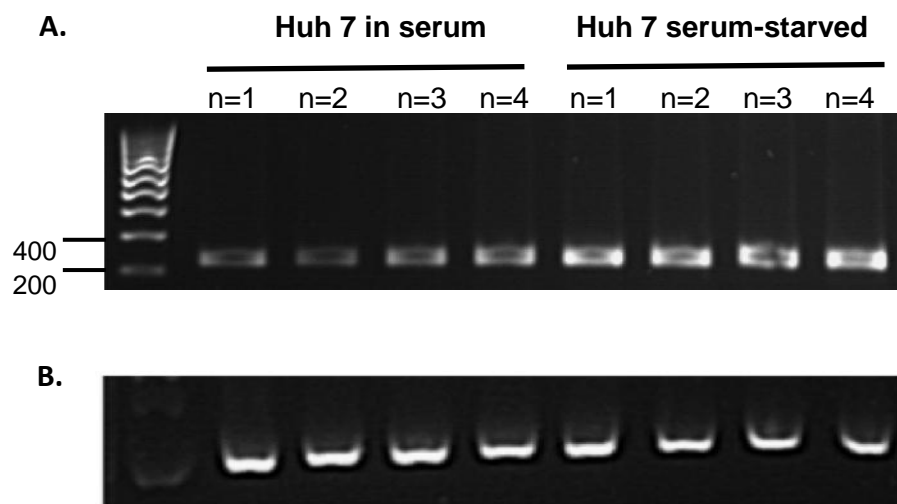
Using non-quantitative PCR, GLP-1R mRNA was inconsistently expressed in traces (35 cycles) in whole donor liver (n=5), BEC (n=5) and explanted primary hepatocytes (n=4), with no evidence in HSEC (n=4) (Figure 7-4). With the exception of whole donor liver (n=10), this was not validated with qPCR. The level of GLP-1R mRNA expression was too low in the whole donor liver samples with qPCR to be accurately quantified against GAPDH. Instead, re-confirmation of GLP-1R presence was confirmed with electrophoresis on agarose gel, with human pancreatic  $\beta$ -cell as the positive control (Figure 7-5). Despite, traces of GLP-1R expression in serum-starved Huh 7 cell lines (subjectively > than non-starved Huh 7) on non-quantitative PCR (Figure 7-6), yet again this was not supported by qPCR.

There was no evidence of GLP-1R protein expression in any liver cell type or whole liver samples. Despite optimising three different polyclonal anti-rabbit primary antibodies against GLP-1R no bands were detected. The quality and the amount of protein loading were confirmed with beta-actin expression.



**Figure 7-4. GLP-1R gene expression in whole liver tissue and liver cell types.**

Non-quantitative PCR (35 cycles) highlighting Inconsistent traces of GLP-1R expression (220 bp) in whole donor liver (non-diseased) **[A]**, primary human hepatocytes **[B]** and biliary epithelial cells (BEC) **[C]**. **[D]** Human sinusoidal endothelial cells (HSEC) did not express the GLP-1R. Reverse transcriptase (RT) negative controls are presented for **[A-D]** and whole donor liver and/or human pancreas were used as positive controls. All experiments were performed three times, on a minimum of 4 separate tissue isolations.



**Figure 7-5. GLP-1R gene expression in Huh 7 cell line.**

Non-quantitative PCR for **[A]** GLP-1R gene expression (220 bp, 35 cycles) in Huh 7 cells cultured with serum-containing (*left*) or serum-free (*right*) media. **[B]** internal standard 18s.

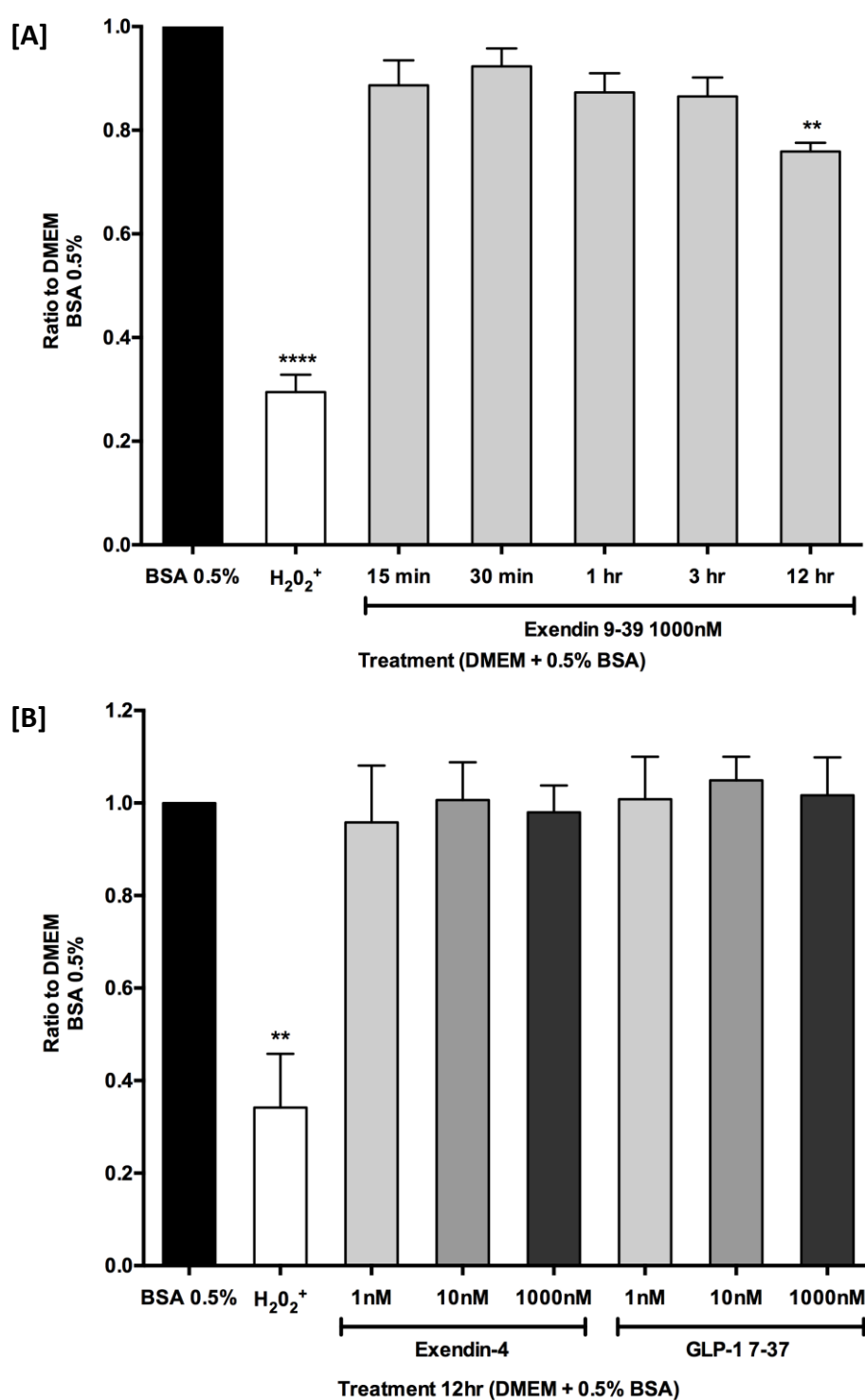


**Figure 7-6. GLP-1R expression in whole donor liver (Qiagen primers)**

Due to the fact that the expression of GLP-1R in whole donor liver [column 2) was too low to quantify via qPCR, GLP-1R gene expression was re-demonstrated on agarose gel using primers from Qiagen (bp 130). Human beta-islet cells acted as the positive control (column 1). No GLP-1R expression was demonstrated in primary hepatocytes [3], Huh 7 cell lines either serum-starved [4] or not [5], HSEC [6] or BEC [7]. GAPDH (bp 199) was the internal standard throughout.

### **7.3.2 GLP-1 effect on Huh 7 cell viability**

Prior to performing functional GLP-1 assays on hepatocytes the effect of different culture medias and doses of GLP-1/exendin-4 on cell viability were determined with MTT assays. Serum-starvation with DMEM BSA 0.5% for 12 hours did not significantly affect Huh 7 cell viability (Figure 7-3). This was therefore the culture medium of choice for the remainder of the experiments. Huh 7 cell line viability was significantly decreased when pre-treated with 1000nM exendin fragment 9-39 (a competitive GLP-1R antagonist) for 12 hours, but not for durations of 15-180 mins (Figure 7-7A). Therefore, for GLP-1R inhibition experiments cells were pre-treated with exendin 9-39 for 30 mins only. Treatment of Huh 7 cells with either exendin-4 (1 to 1000nM) or human GLP-1 7-37 (1 to 1000nM) for 12 hours did not significantly affect the cell viability (Figure 7-7B).



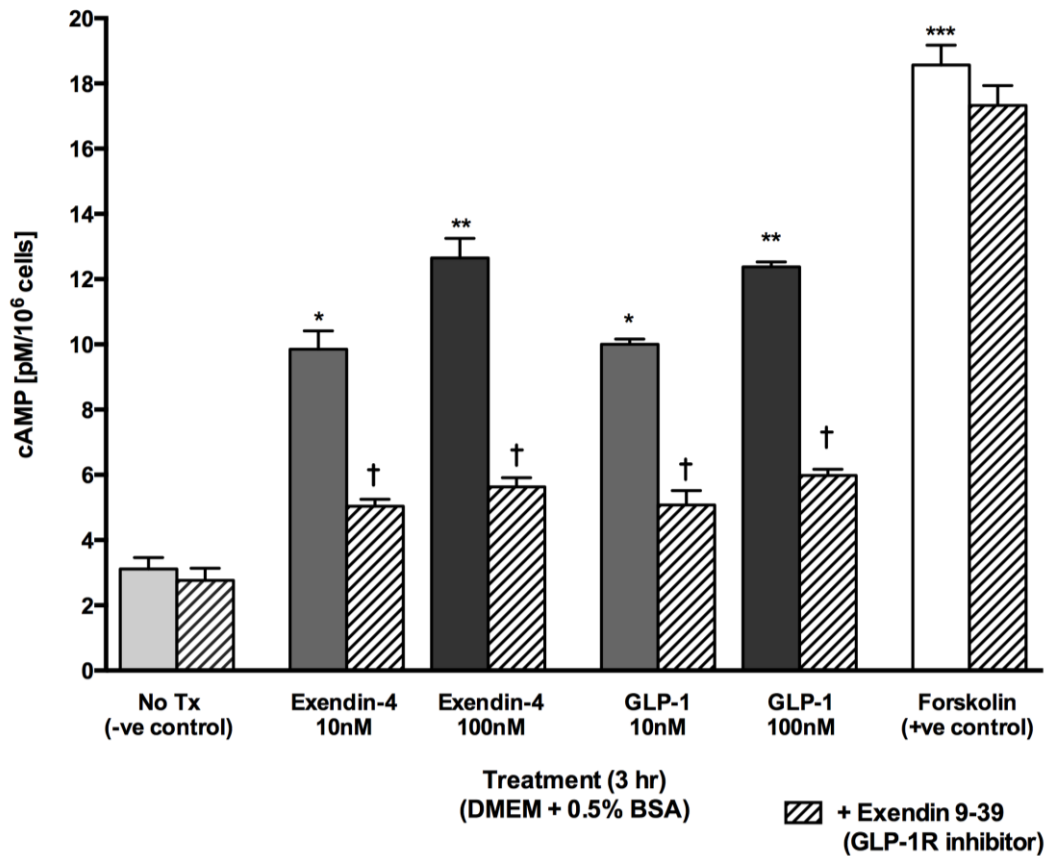
**Figure 7-7. GLP-1 analogues do not effect Huh 7 cell viability**

**[A]** GLP-1R antagonist, exendin 9-39, significantly reduces cell viability compared to non-treated standard serum-starved culture media (DMEM BSA 0.5%) after 12hr incubation, but no effect is seen with variable doses of exendin-4 and GLP-1 7-37 **[B]**. <sup>+</sup>3hr treatment with 2mmol/L hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was used as a positive control for MTT assay. \*\*p<0.01, \*\*\*\*p<0.0001 vs. DMEM BSA 0.5% control (*left column*).



### 7.3.3 GLP-1 effect on G-protein coupled receptor signalling

Three hours treatment with Exendin-4 (10, 100nM) significantly increased intracellular levels of cAMP in Huh 7 cells. Similarly, this effect was replicated with treatment with the active human GLP-1 fragment 7-37, thus highlighting that GLP-1 analogues act directly on liver cells via a G-protein coupled receptor (Figure 7-8).



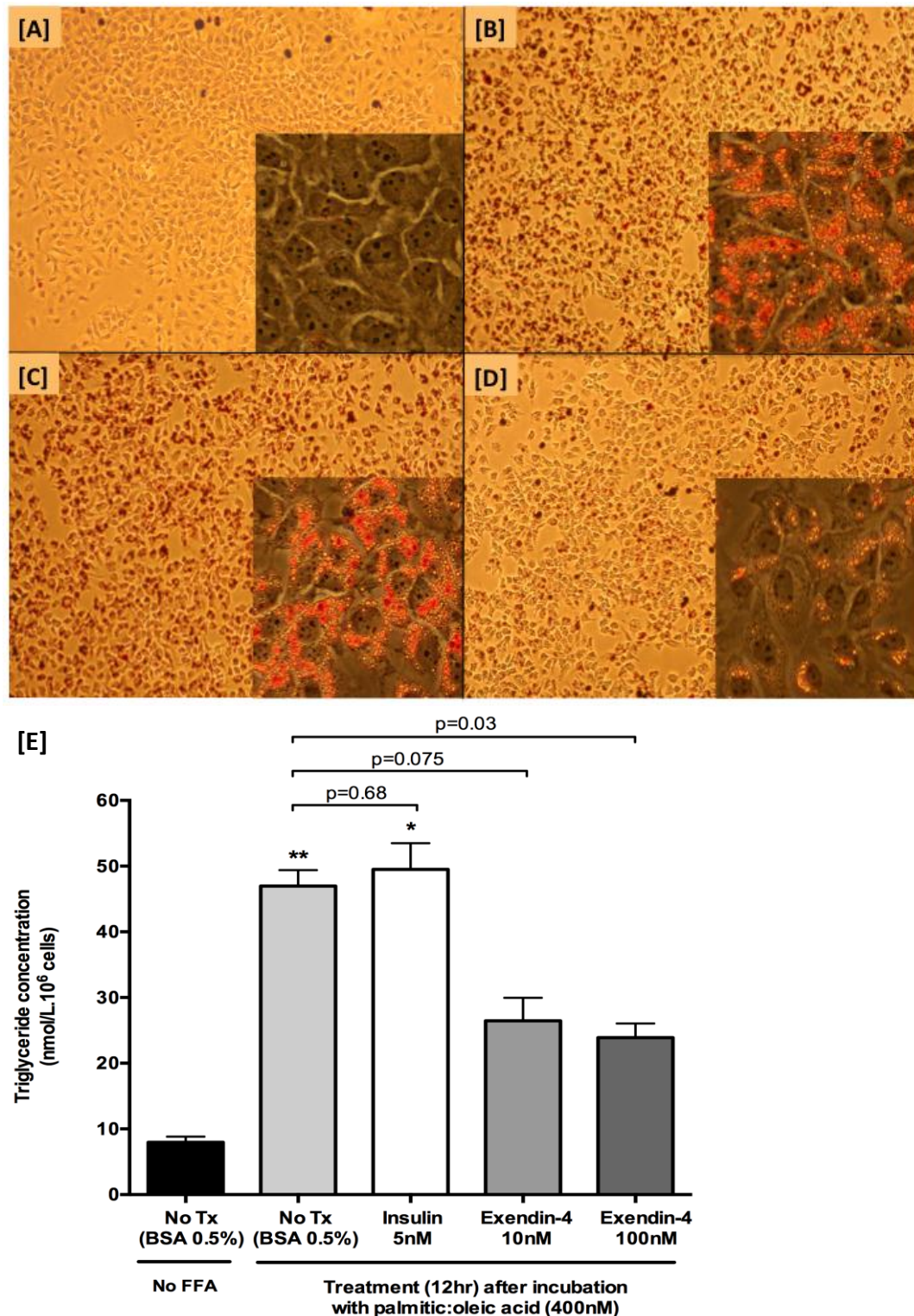
**Figure 7-8. Exendin-4 and human GLP-1 (7-37) significantly increased cAMP production in Huh 7 cells.**

Results are mean $\pm$ SE of cAMP production, expressed in pM per 10<sup>6</sup> Huh 7 cells. Untreated DMEM BSA 0.5% and Forskolin (10  $\mu$ M) served as negative and positive controls, respectively. Exendin fragment 9-39 (competitive GLP-1R antagonist) augmented the effect of GLP-1 analogues on camp production. Experiments were performed three times with each treatment in triplicate. \*  $p$ <0.05, \*\*  $p$ <0.01, and \*\*\*\*  $p$ <0.001 vs. untreated control. †  $p$ <0.05 vs. Exendin 9-39 for each treatment/dose.

The effect of GLP-1 appeared dose-dependent, with greater increases in cAMP production with 100nM than 10nM with both types of GLP-1 treatment. The effect of GLP-1 on cAMP production was significantly inhibited with 30 mins pre-treatment with exendin 9-39, a known competitive antagonist of GLP-1R (Figure 7-8). The magnitude of inhibition of the GLP-1 effect (~50% decrease) with exendin 9-39 was similar with both treatment types and doses.

#### **7.3.4 Anti-steatotic effect of GLP-1**

As seen on Oil Red O staining, the intracellular triglyceride content of Huh 7 cells was markedly increased after incubation with palmitic and oleic acid in the presence/absence of insulin (5nM) compared to the untreated controls (Figure 7-9A-C). 12 hours treatment with exendin-4 (100nM) markedly decreased intracellular triglyceride content, as represented by a reduction in Oil Red O stained droplets (Figure 7-9D). These findings were confirmed with triglyceride quantification using a colorimetric assay (Figure 7-9E). In comparison to the untreated controls, there was a 5-fold increase in the intracellular triglyceride concentration after treatment with palmitic:oleic acid (1:1), compared to untreated controls ( $46.9 \pm 2.4$  vs.  $8.0 \pm 0.9$  nM/ $10^6$  cells;  $p < 0.01$ ). In the presence of insulin (5nM) there was no additional increase in intracellular triglyceride concentration ( $46.9 \pm 2.4$  vs.  $49.5 \pm 4.0$  nM/ $10^6$  cells;  $p > 0.05$ ), indicating that the Huh 7 cells were fully saturated with NEFA loading alone. Exendin-4, at the larger dose of 100nM, significantly reduced the intracellular quantity of triglyceride ( $46.9 \pm 2.4$  vs.  $23.9 \pm 2.2$  nM/ $10^6$  cells;  $p < 0.05$ ). A reduction was also seen with 10nM exendin-4, albeit not significant ( $46.9 \pm 2.4$  vs.  $26.5 \pm 3.5$  nmol/L. $10^6$  cells;  $p = 0.075$ ).



**Figure 7-9. Exendin-4 (100nM) reduces triglyceride content of NEFA-loaded Huh 7 cells**

Oil Red O-stained droplets (red; original magnification x10, x40) are visible in Huh7 cells after treatment (Tx) with NEFA minus insulin [B] & plus insulin [C], compared with untreated cells [A]. Treatment with exendin-4 (100nM) reduces triglyceride content on Oil Red O staining [D] and colorimetric quantification assay [E]. \*p<0.05, \*\*p<0.01 vs. untreated cells.

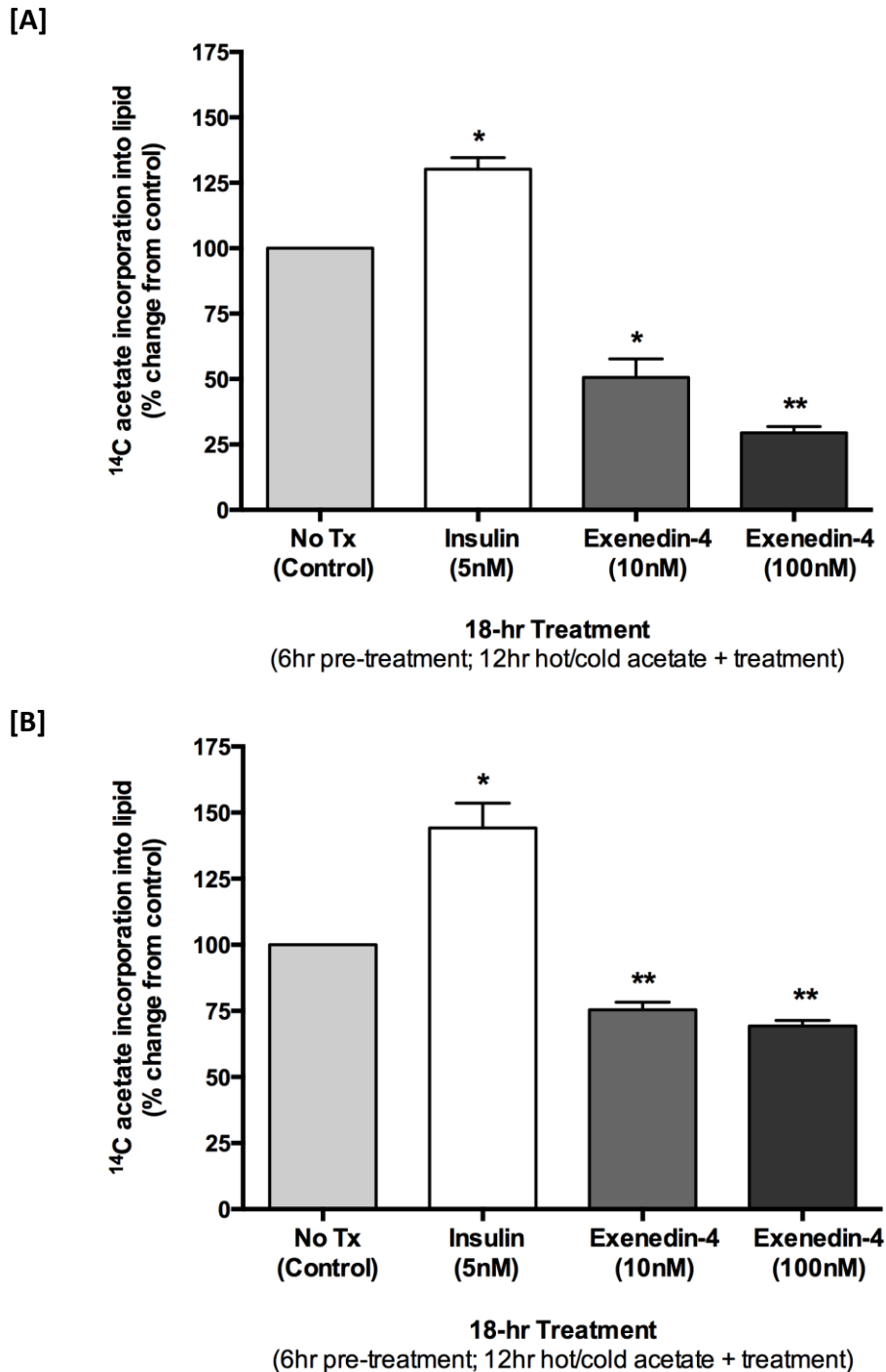
### 7.3.5 GLP-1 effect on DNL in hepatocytes

Exendin-4 (10nM) significantly decreased the amount of  $^{14}\text{C}$ -acetate incorporated into intra-cellular lipid in Huh 7 cells ( $49.4 \pm 7.1$  % decrease vs. control;  $p < 0.05$ ). This was seen to a greater extent with 100nM exendin ( $70.5 \pm 2.4$  % decrease vs. control;  $p < 0.01$ ) (Figure 7-10A). As expected, 5nM insulin significantly increased the amount of  $^{14}\text{C}$ -acetate incorporated into intra-cellular lipid Huh 7 cells ( $30.3 \pm 4.4$  % increase vs. control;  $p < 0.05$ ), representing increased hepatic DNL.

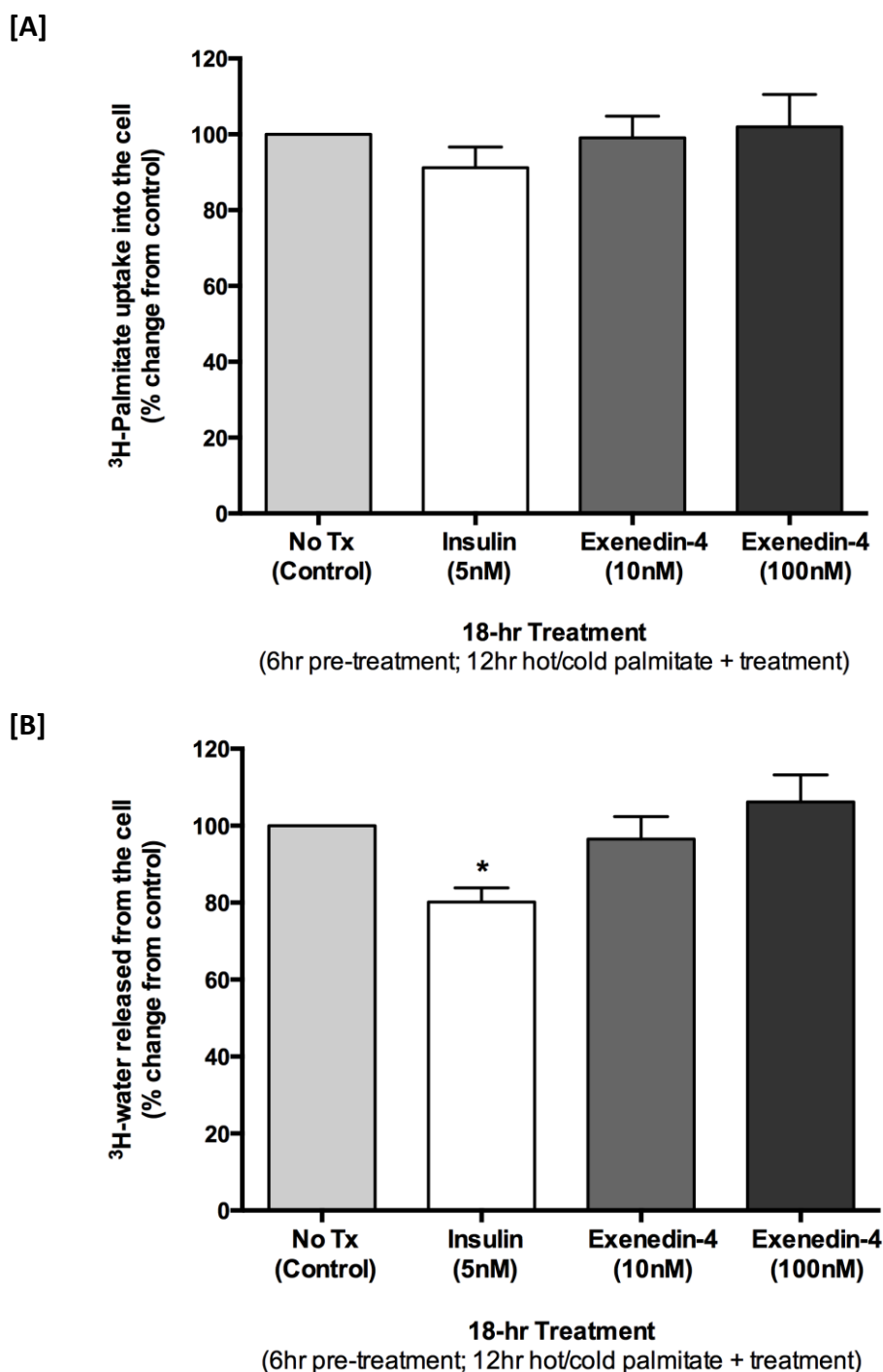
These findings were replicated in primary human hepatocytes ( $n=4$ ), which were isolated from non-obese, non-diabetic male donors (Figure 7-10B). Both 10nM and 100nM Exendin-4 decreased the amount of  $^{14}\text{C}$ -acetate incorporated into intra-cellular lipid in primary hepatocyte cells (10nM:  $24.6 \pm 2.8$ ; 100nM:  $30.7 \pm 2.0$  % decrease vs. control;  $p < 0.01$  for both doses). Collectively, these results highlight that GLP-1 significantly decreases hepatic DNL in hepatocytes.

### 7.3.6 GLP-1 effect on NEFA uptake and $\beta$ -oxidation

NEFA uptake ( $^3\text{H}$ -palmitate) into Huh 7 cells was demonstrated by intracellular  $^3\text{H}$  radioactivity levels between  $1.04\text{--}1.08 \times 10^6$  dpm/ $10^5$  cells in all experiments ( $n=4$ ) and treatment groups (control, insulin, exendin-4). Exendin-4 (10nM or 100nM) had no effect on the amount of NEFA uptake when compared to untreated controls (10nM:  $99.1 \pm 5.7$ ; 100nM:  $101.9 \pm 8.6$  % of controls;  $p > 0.05$  for both doses) (Figure 7-11A).



**Figure 7-10. Exendin-4 significantly reduces DNL in Huh 7 and primary human hepatocytes**  
DNL was defined by the amount of <sup>14</sup>C acetate incorporated into intracellular lipid in Huh 7 cells [A] and primary human hepatocytes [B], after 12 hrs incubation with hot (<sup>14</sup>C) and cold acetate in serum-free media (DMEM BSA 0.5%). Data are presented as mean±SE percentages of the untreated controls. Untreated control was DMEM with 0.5% BSA. Insulin 5nM served as a positive control. Experiments were performed four times with each treatment in quadruplicate. \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. untreated control.



**Figure 7-11. Exendin-4 has no effect on NEFA uptake and  $\beta$ -oxidation in Huh 7 cells**

NEFA uptake was defined by the amount of  $^3\text{H}$ -palmitate taken up by Huh 7 cells after 12 hrs incubation with hot ( $^3\text{H}$ ) and cold palmitate in serum-free media (DMEM BSA 0.5%) [A].  $\beta$ -oxidation was measured by the amount of  $^3\text{H}$  water released by Huh 7 cells into the culture media [B]. Data are presented as mean $\pm$ SE percentages of the untreated controls. Untreated control was DMEM with 0.5% BSA. Insulin 5nM served as a positive control. Experiments were performed four times with each treatment in quadruplicate. \*  $p < 0.05$  vs. untreated control.

The presence of  $\beta$ -oxidation in Huh 7 cells was demonstrated by the cellular release of  $^3\text{H}$  – labelled water (end product of NEFA breakdown) into the culture media, with  $^3\text{H}$  radioactivity levels between  $1.03\text{--}1.31 \times 10^5$  dpm/ $10^5$  cells in all experiments ( $n=4$ ) and treatment groups (control, insulin, exendin-4). As expected, Insulin (5nM) decreased  $\beta$ -oxidation. Exendin-4 (10nM or 100nM) had no effect on  $\beta$ -oxidation when compared to untreated controls (10nM:  $96.5 \pm 5.9$ ; 100nM:  $106.2 \pm 7.0$  % of controls;  $p > 0.05$  for both doses) (Figure 7-11B).

## 7.4 Discussion

In order to fully understand and advance the pharmacological effects of GLP-1 based therapies in patients with NASH it is of paramount importance that studies dissect whether the *in vivo* anti-steatotic effects are solely dependent on weight, glycaemic control or occur via direct GLP-1 signalling in the liver. Even though we found no convincing evidence that human hepatocytes and their neighbouring cells express the GLP-1R, we did demonstrate direct anti-steatotic effects of GLP-1 on hepatocytes *in vitro*. Endorsing our clinical observations in patients with NASH, we found that GLP-1 significantly reduced hepatocyte DNL, but had no effect on NEFA uptake or  $\beta$ -oxidation. In addition, we highlighted intracellular changes consistent with G-protein coupling, which would imply that in the absence of the pancreatic-type GLP-1R that GLP-1 might exert its actions via a second transmembrane receptor. The identification of such a receptor and whether or not subsequent downstream intra-cellular signalling pathways play a role in reversing hepatic steatosis requires further study.

Despite using a variety of established laboratory techniques (standard PCR, qPCR and immunoblots), we failed to provide convincing evidence that primary human hepatocytes express the pancreatic-type GLP-1R. Even though we found very low gene expression in whole human liver samples (obtained from local organ retrieval program), we failed to attribute this to any other liver cell type, including BEC or HSEC. In particular, prior to RNA isolation we starved the cells of any confounding mediators (i.e. dexamethasone, high-dose glucose) that have previously been reported to down-regulate the receptor on pancreatic



cells (Abrahamsen and Nishimura, 1995). As a result of the heterogeneity of gene expression between whole liver samples and the failure to identify the GLP-1R protein with three separate commercial available antibodies, we feel it is unlikely that the GLP-1R exists in the human liver.

Other human data of GLP-1R have emerged in the last 3 years, alongside our study, with conflicting results (Aviv et al., 2009; Gupta et al., 2010; Svegliati-Baroni et al., 2011; Lee et al., 2012; Panjwani et al., 2013). In support of our data, Aviv and colleagues failed to identify the receptor in primary hepatocytes isolated from both children and adults over 40 years of age (Aviv et al., 2009). In contrast, Gupta et al reported the presence of the GLP-1R in human hepatocytes using a wide range of techniques (i.e. PCR, immunoblots, immunofluorescence with confocal microscopy, and bioluminescence) and highlighted that the GLP-1R is internalised on activation with the ligand in Huh 7 cell lines (Gupta et al., 2010). However, they provided very little data on the culture duration and origin of the primary hepatocytes used, which in turn might alter gene expression. Furthermore, there was no justification as to why primary hepatocytes were only used for PCR and western blotting and not for the more robust staining techniques, in which hepatoma cell lines were used instead. Perhaps the most robust published study to date is that by Panjwani and colleagues, in which they went to extensive efforts to validate the previously reported identification techniques (Panjwani et al., 2013). Contrary to the reports by Gupta et al, they found no evidence of GLP-1R mRNA in well characterised primary hepatocytes, despite using primer pairs that spanned the entire GLP-1R open reading frame (Panjwani et al., 2013). Furthermore, Panjwani et al assessed the sensitivity (i.e. on cells transfected with the GLP-1R cDNA) and

specificity (i.e. on lung tissue from GLP-1R  $-/-$ ) of the three commercially available GLP-1R antibodies that were used in our study. Despite using a highly sensitive immunoprecipitation technique on lung tissue from wild type mice (positive control) and GLP-1R  $-/-$  mice (negative control), they found vague protein bands for the GLP-1R in both types of tissue, which is highly suggestive of false positive staining (Panjwani et al., 2013). Knudson and colleagues have since corroborated these findings in human tissue (Pyke and Knudsen, 2013) and proceeded to recommend the use of fluorescence-labelled GLP-1 analogues (Reiner et al., 2011) *in vivo* or *ex vivo* for future receptor identification.

Even though there was no GLP-1R expression, we clearly highlighted activation of intracellular cAMP signalling (3-4 fold increase) in the presence of both native GLP-1(7-37) amide and exendin-4. We performed these experiments in Huh 7 hepatoma cell lines (i.e. readily available and cost-effective), however, other groups have replicated our findings in primary human hepatocytes in the absence of GLP-1R (Aviv et al., 2009). Somewhat surprisingly in our study, we found that the GLP-1R antagonist, exendin (9-39), inhibited the effect of GLP-1 on cAMP signalling. However, as the effect was only partially inhibited (50%), it would imply that GLP-1 is potentially interacting with other G-protein coupled receptors on the cell surface of hepatocytes. This remains a controversial and complex field, but there is a growing body of evidence to suggest that a second type of GLP-1R may exist (*unidentified at present*) or that GLP-1 enters the cell via alternative mechanisms (Tomas and Habener, 2010). Indeed, recent studies have shown that the DPP-4 breakdown product of GLP-1 (GLP-1(9-36) amide), which has a very low affinity for the pancreatic-type GLP-1R, reverses hepatic steatosis in mice fed a HFD (Fat 60% of calories) (Tomas et al., 2011b). In

addition, studies have shown cardioprotective properties of the GLP-1(9-36) amide in rodent and canine experiments, which are independent to GLP-1R (Ban et al., 2008). Some investigators have attempted to utilise the GLP-1R knockout mice (GLP-1R  $-/-$ ) to investigate whether the metabolic effects of GLP-1 are independent to its receptor (Trevaskis et al., 2012). However, as is commonly reported with transgenic models, compensatory mechanisms might occur (i.e. GIP release), which would significantly limit the meaning of any findings (Ayala et al., 2010).

Importantly, using several *in vitro* experimental methods, we have highlighted that GLP-1 based therapies (exendin-4) have direct anti-steatotic effects on the human hepatocytes. After fat loading the hepatocytes *in vitro*, we demonstrated that exendin-4 (in the absence of insulin) decreases intracellular lipid stores, as seen by histological staining and assay quantification. This has since been replicated in both hepatoma cell lines and primary human hepatocytes under similar culture conditions (Gupta et al., 2010; Sharma et al., 2011; Lee et al., 2012). Paralleling their *ex vivo* rodent findings, several groups have demonstrated changes in hepatic gene expression in GLP-1 treated hepatocytes consistent with decreased hepatic DNL ( $\downarrow$  ACC1, FAS, SCD-1, SREBP-1C) and increased  $\beta$ -oxidation ( $\uparrow$  CPT1A, PPAR $\alpha$ ) (Ding et al., 2006; Ben-Shlomo et al., 2011; Gu et al., 2011; Svegliati-Baroni et al., 2011). However, as these findings solely relied on single time-point analyses of hepatic gene ( $\pm$  protein) expression pre/post GLP-1 therapy, we endeavored to determine the functional significance of these changes in hepatocyte lipid handling.

Using state-of-the art isotope tracer studies we found that exendin-4 significantly reduced hepatic DNL in hepatoma cell lines. Importantly, endorsing our clinical observations in patients with NASH (Chapter 6), we replicated these findings using well-characterised primary human hepatocytes from donors who had no prior metabolic complications. This therefore highlights that the anti-DNL effects of GLP-1 based therapies can occur independent of weight loss and is highly suggestive that some (but maybe not all) of their action is dependent upon a direct action on the liver. Furthermore, in an attempt to mimic the fasting state of our human studies, our *in vitro* experiments were performed after prolonged serum-starvation and in the absence of insulin (with exception of positive controls), implying that GLP-1's anti-steatotic actions *in vitro* were not insulin-mediated. In contrast to the previous gene expression studies, we failed to demonstrate a functional effect of GLP-1 on NEFA uptake and breakdown in hepatocytes using robust radio-labelled palmitate protocols. Even though we have not replicated this specific finding in primary hepatocytes, it does imply that the majority of the anti-steatotic effect of GLP-1 in hepatocytes is via DNL, rather than NEFA breakdown. Whether this holds true for hepatocytes that are under lipotoxic stress (vs. NEFA free conditions), requires further study. Taking this further, Sharma et al have recently shown that GLP-1 based therapies appear to protect hepatocytes that have been loaded with NEFA from oxidative (endoplasmic reticulum) stress and subsequent cellular death *in vitro*. This direct effect of GLP-1 on hepatocyte apoptosis is in keeping with previous studies in pancreatic  $\beta$ -cells (Cunha et al., 2009). It is important to acknowledge that although these *in vitro* experiments offer important insights into the direct effect of GLP-1 on cells, they are not truly representative

of the complex hormonal and inflammatory milieu, which is evident *in vivo* in patients with NASH.

Even though we have shown that GLP-1 interacts with hepatocytes via G-protein coupling and subsequent cAMP production, it was beyond the aims of the current project to determine whether associated down-stream signal transduction pathways are linked to the decreases in DNL. Even though several mechanisms have recently been proposed (Hou et al., 2008; Aviv et al., 2009; Gupta et al., 2010; Ben-Shlomo et al., 2011; Samson et al., 2011; Svegliati-Baroni et al., 2011; Lee et al., 2012), it remains far from clear as to which is the dominant pathway. Gupta et al have recently shown that GLP-1 may exert its anti-steatotic effect by interacting with the insulin signaling pathway, via marked activation of 3-phosphoinositide-dependent kinase-1 (PDK-1) and key downstream protein kinases (namely, Akt/PKB and PKC) (Gupta et al., 2010). Others have in part supported this in the field (Aviv et al., 2009; Svegliati-Baroni et al., 2011). Others have studied the role of AMP-activated protein kinase (AMPK), which is important in regulating the energy state of hepatocytes and has repeatedly been shown to be activated (phosphorylated) in GLP-1 treated hepatocytes (Samson et al., 2008; Ben-Shlomo et al., 2011; Svegliati-Baroni et al., 2011; Lee et al., 2012). Ben-Sholmo et al and others have highlighted that AMPK inactivates ACC1, in addition to FAS, which are key rate limiting enzymes in hepatic DNL (Samson et al., 2008; Ben-Shlomo et al., 2011). This is supported by the fact that AMPK deficient murine models have marked increased hepatic expression of these lipogenic enzymes (Shaw et al., 2005). In addition AMPK has been shown to augment  $\beta$ -oxidation (Ben-Shlomo et al., 2011), but at present our *in vitro* data questions the functional effects of such in response to GLP-1 in the absence of

metabolic confounders. It is also important to note that cAMP-activated protein kinase is a different entity to AMPK and shouldn't be confused (Samson and Bajaj, 2013). Therefore, there is no robust evidence at present to link GLP-1's effect on intracellular cAMP levels and its ability to activate AMPK. Overall, a better understanding of how GLP-1 and its analogues induce down stream activation of AMPK is still required.

In summary, we have demonstrated that GLP-1 based therapies have direct anti-lipogenic effects on hepatocytes in the absence of GLP-1R detection. It is plausible that GLP-1 initiates these effects via a second transmembrane receptor on hepatocytes, which has the characteristics of a G-protein coupling receptor. Our data support the notion that the anti-steatotic effects of GLP-1 are not solely reliant on improvements in weight and/or glycaemic control. This might have important therapeutic implications for normoglycaemic, lean patients with NASH.

## **CHAPTER 8: GENERAL DISCUSSION AND CONCLUDING REMARKS**

NAFLD is an alarming global public health problem, which is predicted to increase in parallel to the exponential rise in the incidence of type 2 diabetes and obesity. In particular, NASH  $\pm$  advanced fibrosis incurs a significantly increased risk of both liver- and CVD-related morbidity and death. Despite the fact that our knowledge of the pathogenesis, diagnosis and prognosis of NASH has expanded over the last decade, there is still no universally accepted safe, pharmacological therapy for NASH. The studies presented here have targeted some of the critical gaps in our knowledge with regards to: a) the severity of NAFLD in primary care, b) tissue-specific IR and lipotoxicity, and most notably c) identification of a novel therapeutic option in patients with NASH. These are summarised below, with recent updates in the field and suggestions for future research.

### **8.1 Clinical burden of NAFLD and advanced fibrosis in primary care**

Due to the lack of simple and cost-effective diagnostic markers of NAFLD in the past, understanding the true burden of NAFLD in primary care has been a challenge. However, with recent advances in non-invasive biomarkers and in particular the development of simple and applicable scoring systems (i.e. NFS, Fib-4) this has now become feasible. Utilising a large prospective cohort of over 1000 patients, we found that NAFLD was the commonest cause (26%; 295/1118) of incidental abnormal LFTs in primary care. In contrast, other classical causes of liver disease (including HCV, HBV, haemochromatosis) accounted for less than 3%. In addition to its prospective design, one of the main strengths of this work was the

high proportion (98%) of the participants that successfully underwent a thorough diagnostic work-up for liver disease, including alcohol history, USS and full serological aetiology screen. Prior to our study, data regarding the prevalence of advanced fibrosis, which incurs the highest risk of HCC, liver failure and death, was absent in the community setting. By applying the easy-to-use and cheap NFS (*1<sup>st</sup> time in this clinical setting*) we estimated that 7.6% of patients with USS-defined NAFLD patients have advanced fibrosis (F3/F4) (Armstrong et al., 2012). This figure is particularly concerning, in light of the fact that the study population consisted of patients whom had no prior history, signs or specific symptoms of liver disease. Furthermore, the finding of an abnormal LFT was incidental, as the vast majority of the bloods were ordered as part of routine 'chronic' health check ups. None the less, it must be noted that over one-third of our cohort had diabetes, obesity or hypertension; all of which are now known metabolic risk factors for NAFLD.

The most notable limitations of our study were the fact that it did not include patients with normal LFTs and that the NFS was designed and validated in secondary care populations, which are open to selection bias. However, studies that have emerged at the same time as ours, albeit from a variety of communities, have reported similar rates of advanced fibrosis in patients with and without abnormal LFTs (3 to 7%) (Poynard et al., 2010; Williams et al., 2011; Wong et al., 2012a). The variation between studies is likely due to differences in study population (e.g. Japanese community vs. US medical centre) and the fibrosis marker utilised (i.e. transient elastography, Fibrotest). Perhaps the closest study to ours was that performed in a US medical army barracks in 2011, in which 7% of the army personal whom underwent an USS and liver biopsy (*after presenting with non-specific reasons*) were found to have



histological evidence of advanced fibrosis (Williams et al., 2011). Even though such an approach would be deemed too invasive and in parts unethical in the UK, data from Williams et al does highlight that our estimation is likely accurate (Williams et al., 2011).

The original data collection for our study took place over 7-8 years ago in primary care. Due to significant advances in non-invasive markers of hepatic steatosis and fibrosis in this period, it is an opportune time to follow this unique cohort up. By doing so, it would answer some important questions. First, as it is unlikely without intervention that fibrosis would have regressed in this time; the validity of the original NFS result could be assessed with more complex fibrosis markers (e.g. transient elastography, ELF; *acknowledging the limitations of such*). Second, did the diagnosis of NAFLD (with/without advanced fibrosis) in primary care result in the development of significant liver and non-related liver morbidity (especially CVD, incident diabetes)? Lastly, what was the clinical burden (i.e. hospitalisation, primary care appointments) and impact on mortality in those with NAFLD? This question is particularly relevant in light of an interesting publication by Angulo et al., in which they retrospectively assessed the performance of NFS (and other scores) as predictors of liver-related morbidity, transplantation and death in 320 patients from secondary care (Angulo et al., 2013). The median follow-up was 8.5 years and as with our study, participants were predominantly Caucasian. NFS was found to be the best predictor of liver-related morbidity, death/transplant and CVD-mortality, with significantly higher adjusted hazard ratios in those that had intermediate/high NFS (N.B. high 5x > intermediate) compared to those with a low score. Kim et al have reported similar findings in over 11,000 patients with fibrosis rates similar to our cohort. Even though they failed to show a relationship with liver-related death

(largely due to small numbers with such), NFS predicted overall mortality during their 14 year follow-up study (Kim et al., 2013a). On this basis, it would be very interesting to assess the predictive value of NFS for clinical outcomes in our primary care cohort. A finding of such would potentially aid in prioritising those in greatest need of specialist liver input, clinical trial participation and most importantly optimisation of CVD risk profile.

## **8.2 Adipose tissue IR and lipotoxicity in patients with NASH – abdominal SAT is more than just a bystander**

For over a decade, systemic IR has been widely recognised as one of the main pathological features of NASH (Marchesini et al., 2001; Chitturi et al., 2002). However, prior to designing my PhD less than a handful of studies had investigated the relative contribution of tissue-specific insulin sensitivity in patients with biopsy-proven NASH (Marchesini et al., 2001; Sanyal et al., 2001; Bugianesi et al., 2005a). Furthermore, very little in-situ data existed on the contribution of dysfunctional adipose tissue in human NASH.

By adopting an integrative physiological approach with functional measures of lipid and carbohydrate flux (using ‘gold-standard’ euglycaemic clamp and isotope technology), we demonstrated that patients with NASH (vs. healthy controls) have marked adipose dysfunction, alongside increased hepatic and muscle IR. This finding was corroborated by parallel work undertaken by external research groups in the US and Italy (Gastaldelli et al., 2009; Lomonaco et al., 2011; Lomonaco et al., 2012; Musso et al., 2012). These studies, however, solely relied on circulating NEFA and insulin levels to evaluate whole-body adipose

insulin sensitivity (majority in the fasting state only). In contrast, we demonstrated severe adipose IR using several modalities. Most importantly, by measuring in-situ glycerol release (a product of lipolysis), we reported novel insights into the severity of IR and lipolysis in localised abdominal SAT of patients with NASH. Indeed the magnitude of the difference between IR in whole-body adipose tissue and localised abdominal SAT implies that the latter is more than just a bystander to VAT in patients with NASH. As a result, we have hypothesised that abdominal SAT may act as an important buffer for chronic energy supply, which in the case of NASH, fails to cope with the demand. Subsequently, fat-derived toxic metabolites flux from the abdominal SAT to the neighbouring VAT and to the liver (via the portal vein), in which the lipotoxicity initiates a cascade of liver injury.

Due to the limitations of our study, which we have previously eluded to (i.e. unmatched control arm), it is now important to investigate whether these localised adipose findings are independent of obesity. This could be achieved with a variety of study designs, including: a) investigating non-obese, normoglycaemic patients with NASH (*acknowledging that recruitment would be challenging*); b) larger sample size to enable adjustment for metabolic confounders (*potentially costly*); or c) incorporate measures of VAT/SAT mass and volume to adjust for a tissue-mass effect (using MRI). Furthermore, future studies should not only aim to assess in-situ fluxes in lipid, but should analyse the genetic and proteomic profiles of adipose depots (via biopsy) in lean and obese patients with NASH. Indeed, we intend to perform proteomics on the remaining SAT microdialysis fluid from our study. By investigating what makes NASH patients susceptible to 'sick' fat tissue, might aid with therapeutic targets for the future.

### **8.3 Beneficial effect of GLP-1 based therapies in patients with NASH**

In light of the above, it is not surprising that insulin sensitisers have been employed by the majority of RCTs in NASH to date. Unlike other anti-diabetic drugs, which have a tendency to promote weight gain (i.e. TZDs), GLP-1 based therapies induce weight loss in addition to improving glucose homeostasis in patients with type 2 diabetes. These actions, together with data from human case reports (Tushuizen et al., 2006; Ellrichmann et al., 2009) and various animal models of NAFLD (Ding et al., 2006; Lee et al., 2007), led to the current hypothesis that pharmaceutical GLP-1 agents could be a novel therapeutic option in NASH. Therefore, we investigated the role of GLP-1 in three stages: a) retrospective analysis to assess safety and efficacy in patients with abnormal liver enzymes; b) prospective RCT to investigate the metabolic and histological effects of GLP-1 in patients with NASH; and c) in vitro study to assess the direct actions of GLP-1 in human hepatocytes.

#### **8.3.1 Liraglutide (GLP-1 analogue) is well tolerated, safe and improves liver enzymes in overweight patients type 2 diabetes**

Due to a paucity of data, regulatory authorities have previously issued caution against the use of GLP-1 agents with patients with mild, moderate and severe liver injury. By performing a large-scale meta-analysis of over 4000 patients with type 2 diabetes we highlighted that short-term treatment (6 months) with 1.8mg liraglutide has an acceptable safety profile and significantly improves liver enzymes in patients with asymptomatic liver injury (Armstrong et al., 2013d). The effect on both liver enzymes (meta-analysis) and hepatic steatosis (CT sub-

study) appeared to be dose-dependent (1.8mg > 1.2mg) and after adjustment for confounders appeared to be mediated by weight loss and glycaemic control. Albeit retrospective, this study was the first large-scale study to utilise individual patient data and report comparisons to placebo-control. Since our work, two small, uncontrolled studies have supported our findings, with reported reductions in hepatic steatosis on H-MRS (n=19 exenatide; n=6 liraglutide) and liver biopsy (n=8 exenatide) (Kenny et al., 2010; Cuthbertson et al., 2012). The latter case series also reported improvements in inflammation and hepatocyte ballooning, but their small sample size and lack of control restricted any further conclusions. Collectively, these data highlighted that the actions of GLP-1 agents, in particular liraglutide, warranted prospective controlled study.

### **8.3.2 Liraglutide reduces adipose IR, inflammation and hepatic DNL in patients with NASH**

Therapies that rescue the liver from the effects of lipotoxicity are believed to be essential for preventing the progression of NASH to end-stage disease (Cusi, 2012). Our prospective, double-blinded randomised-control study highlighted that 1.8mg liraglutide improves key components of lipotoxic injury in NASH. Using state-of-the-art euglycaemic clamp/isotope tracer techniques our study represented the first *in vivo* description of the beneficial effects of liraglutide on hepatic lipogenesis, hepatic IR, and most notably adipose IR, as represented by improvements in both whole-body and localised abdominal lipolysis. The subsequent reductions in circulating NEFA and pro-inflammatory mediators (most notably leptin and CCL-2), likely explain the improvements we saw in IR and lipid handling in the liver (Kashyap

et al., 2003; Boden, 2006). This was further evident by the significant reductions we witnessed in liver enzymes, HbA1c and LDL-cholesterol. Collectively, our robust metabolic findings not only predicted that 1.8mg liraglutide might result in histological improvements in NASH, but by reducing lipotoxicity and known risk factors (obesity, hyperglycaemia, hyperlipidaemia) may also reduce the long-term risk of cardiovascular morbidity. Indeed, the latter is currently the focus of a large-scale placebo-controlled RCT in 9,000 patients with type diabetes, known as the LEADER trial ([clinicaltrials.gov NCT01179048](https://clinicaltrials.gov/ct2/show/study/NCT01179048)), which is due to finish in 2016. Even though there are no other human studies in NASH to compare our findings too at present, our data translates findings from several murine studies of GLP-1 in the literature (Lee et al., 2007; Shirakawa et al., 2011; Lee et al., 2012; Zhang et al., 2013).

Of notable interest, is the fact that improvements in circulating adiponectin and adipose IR index, reported here, have previously been implicated in the resolution of lipotoxic liver injury and fibrosis in humans (Xu et al., 2003; Gastaldelli et al., 2009; Gastaldelli et al., 2010). Even though we can only speculate at present, this provides further evidence that liraglutide might result in histological improvements in NASH. The answer to such awaits the completion of our phase II, multi-centre RCT in May 2014; which has successfully recruited to the calculated target of 50 patients. If the trial were to conclude that liraglutide is safe and warrants further histological analysis in patients with NASH, the aim would be to design a phase III RCT.

### **8.3.3 GLP-1 based therapies have direct anti-lipogenic (weight independent) effects on hepatocytes in vitro**

Investigating the potential mechanisms of GLP-1 agents is of paramount importance to understanding their full therapeutic potential. Our in-vitro observations support the notion that the anti-steatotic effects of GLP-1 are not solely reliant on improvements in weight and/or glycaemic control. Using state-of-the-art isotope tracer methods, we found that GLP-1 reduced DNL in primary human hepatocytes, thus mirroring the effect we observed in our trial participants. This is supported by positive findings in weight neutral rodents studies (Gedulin et al., 2005; Shirakawa et al., 2011; Trevaskis et al., 2012) and the reduced gene expression of key enzymes involved in hepatocyte DNL after GLP-1 treatment in culture (Ding et al., 2006; Ben-Shlomo et al., 2011; Gu et al., 2011; Svegliati-Baroni et al., 2011). In addition, we highlighted intracellular changes consistent with G-protein coupling, which would imply that in the absence of the pancreatic-type GLP-1R, that GLP-1 might exert its actions via a second transmembrane receptor. The identification of such a receptor and whether or not subsequent downstream intra-cellular signalling pathways play a role in reversing hepatic steatosis and IR requires further study.

Furthermore, we plan to expand on our human experiments by investigating the direct effect of GLP-1 on human adipose tissue. Several studies to date have reported that GLP-1 increases insulin sensitivity in adipocytes, however this data is restricted to 3T3 adipocyte cell lines only (Egan et al., 1994; Wang et al., 1997; Gao et al., 2007; Vendrell et al., 2011). Isolation of human adipocytes from paired samples of SAT and VAT will enable us to

investigate the depot-specific effects of GLP-1 in culture. Taking this further, our laboratory has optimised the technique of co-culturing adipocytes and hepatocytes, which will enable us to begin to investigate how GLP-1 might effect the 'cross-talk' between these two influential cell types, in the absence of other metabolic confounders.

## **8.4 Conclusion**

Our findings highlight that NAFLD is the commonest cause of liver disease in UK primary care, yet a significant proportion with advanced fibrosis remain undetected. Those patients with biopsy confirmed NASH were found to have marked multi-organ IR and pathological levels of circulating adipose-derived toxic metabolites, the majority of which appear to originate from abdominal SAT. Reducing lipotoxicity appears key in the management of NASH.

GLP-1 based therapies appear to be safe and well tolerated in patients at risk of underlying NAFLD. Our prospective controlled study has highlighted that the long-acting GLP-1 analogue, liraglutide, significantly reduces metabolic dysfunction, hepatic lipogenesis, hepatic/adipose IR and adipose inflammation in patients with NASH. Our In-vitro studies indicate that the anti-steatotic effects are not solely reliant on improvements in weight and/or glycaemic control. Taken together, our findings highlights that GLP-1 based therapies have all the metabolic and clinical attributes to make them a promising therapeutic option in patients with NASH. However, the safety and histological efficacy of such awaits the



completion of our 48-week RCT (LEAN trial), which is integral to whether or not larger studies are warranted.

## REFERENCES

- A'HERN, R. P. 2001. Sample size tables for exact single-stage phase II designs. *Stat Med*; 20: 859-66.
- ABATE, N., GARG, A., PESHOCK, R. M., STRAY-GUNDERSEN, J., ADAMS-HUET, B. & GRUNDY, S. M. 1996. Relationship of generalized and regional adiposity to insulin sensitivity in men with NIDDM. *Diabetes*; 45: 1684-93.
- ABATE, N., GARG, A., PESHOCK, R. M., STRAY-GUNDERSEN, J. & GRUNDY, S. M. 1995. Relationships of generalized and regional adiposity to insulin sensitivity in men. *J Clin Invest*; 96: 88-98.
- ABDELMALEK, M. F., SANDERSON, S. O., ANGULO, P., SOLDEVILA-PICO, C., LIU, C., PETER, J., KEACH, J., CAVE, M., CHEN, T., MCCLAIN, C. J. & LINDOR, K. D. 2009. Betaine for nonalcoholic fatty liver disease: results of a randomized placebo-controlled trial. *Hepatology*; 50: 1818-26.
- ABRAHAMSEN, N. & NISHIMURA, E. 1995. Regulation of glucagon and glucagon-like peptide-1 receptor messenger ribonucleic acid expression in cultured rat pancreatic islets by glucose, cyclic adenosine 3',5'-monophosphate, and glucocorticoids. *Endocrinology*; 136: 1572-8.
- ADAMS, L. A., BULSARA, M., ROSSI, E., DEBOER, B., SPEERS, D., GEORGE, J., KENCH, J., FARRELL, G., MCCAUGHAN, G. W. & JEFFREY, G. P. 2005a. Hepascore: an accurate validated predictor of liver fibrosis in chronic hepatitis C infection. *Clin Chem*; 51: 1867-73.
- ADAMS, L. A., GEORGE, J., BUGIANESI, E., ROSSI, E., DE BOER, W. B., VAN DER POORTEN, D., CHING, H. L. I., BULSARA, M. & JEFFREY, G. P. 2011. Complex non-invasive fibrosis models are more accurate than simple models in non-alcoholic fatty liver disease. *J Gastroenterol Hepatol*; 26: 1536-43.
- ADAMS, L. A., LYMP, J. F., ST SAUVER, J., SANDERSON, S. O., LINDOR, K. D., FELDSTEIN, A. & ANGULO, P. 2005b. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology*; 129: 113-21.

- ADAMS, L. A., SANDERSON, S., LINDOR, K. D. & ANGULO, P. 2005c. The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J Hepatol*; 42: 132-8.
- AHMAD, S. R. & SWANN, J. 2008. Exenatide and rare adverse events. *N Engl J Med*; 358: 1970-1; discussion 1971-2.
- AHMED, M. H., ABU, E. O. & BYRNE, C. D. 2010. Non-Alcoholic Fatty Liver Disease (NAFLD): new challenge for general practitioners and important burden for health authorities? *Prim Care Diabetes*; 4: 129-37.
- AITHAL, G. P., THOMAS, J. A., KAYE, P. V., LAWSON, A., RYDER, S. D., SPENDLOVE, I., AUSTIN, A. S., FREEMAN, J. G., MORGAN, L. & WEBBER, J. 2008. Randomized, placebo-controlled trial of pioglitazone in nondiabetic subjects with nonalcoholic steatohepatitis. *Gastroenterology*; 135: 1176-1184.
- ALVES, C., BATEL-MARQUES, F. & MACEDO, A. F. 2012. A meta-analysis of serious adverse events reported with exenatide and liraglutide: acute pancreatitis and cancer. *Diabetes Res Clin Pract*; 98: 271-84.
- AMIR, M. & CZAJA, M. J. 2011. Autophagy in nonalcoholic steatohepatitis. *Expert Rev Gastroenterol Hepatol*; 5: 159-66.
- AMISTEN, S. 2012. A rapid and efficient platelet purification protocol for platelet gene expression studies. *Methods Mol Biol*; 788: 155-72.
- ANGELICO, F., BURATTIN, M., ALESSANDRI, C., DEL BEN, M. & LIRUSSI, F. 2007. Drugs improving insulin resistance for non-alcoholic fatty liver disease and/or non-alcoholic steatohepatitis. *Cochrane Database Syst Rev*: CD005166.
- ANGULO, P. 2010. Long-term mortality in nonalcoholic fatty liver disease: is liver histology of any prognostic significance? *Hepatology*; 51: 373-5.

ANGULO, P., BUGIANESI, E., BJORNSSON, E. S., CHARATCHAROENWITTHAYA, P., MILLS, P. R., BARRERA, F., HAFLIDADOTTIR, S., DAY, C. P. & GEORGE, J. 2013. Simple Noninvasive Systems Predict Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology*; 145: 782-789.

ANGULO, P., HUI, J. M., MARCHESINI, G., BUGIANESI, E., GEORGE, J., FARRELL, G. C., ENDERS, F., SAKSENA, S., BURT, A. D., BIDA, J. P., LINDOR, K., SANDERSON, S. O., LENZI, M., ADAMS, L. A., KENCH, J., THERNEAU, T. M. & DAY, C. P. 2007. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology*; 45: 846-54.

ANSTEE, Q. M., TARGHER, G. & DAY, C. P. 2013. Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. *Nat Rev Gastroenterol Hepatol*; 10: 330-44.

ARAKAWA, M., EBATO, C., MITA, T., HIROSE, T., KAWAMORI, R., FUJITANI, Y. & WATADA, H. 2009. Effects of exendin-4 on glucose tolerance, insulin secretion, and beta-cell proliferation depend on treatment dose, treatment duration and meal contents. *Biochem Biophys Res Commun*; 390: 809-14.

ARGO, C. K. & CALDWELL, S. H. 2009. Epidemiology and natural history of non-alcoholic steatohepatitis. *Clin Liver Dis*; 13: 511-31.

ARGO, C. K., NORTHUP, P. G., AL-OSAIMI, A. M. S. & CALDWELL, S. H. 2009. Systematic review of risk factors for fibrosis progression in non-alcoholic steatohepatitis. *Journal of Hepatology*; 51: 371-9.

ARMSTRONG, M. J., ADAMS, L. A., CANBAY, A. & SYN, W. K. 2013a. Extra-hepatic complications of nonalcoholic fatty liver disease. *Hepatology* [Epub ahead of print].

ARMSTRONG, M. J., CORBETT, C., HODSON, J., MARWAH, N., PARKER, R., HOULIHAN, D. D., ROWE, I. A., HAZLEHURST, J. M., BROWN, R., HÜBSCHER, S. G. & MUTIMER, D. 2013b. Operator training requirements and diagnostic accuracy of Fibroscan in routine clinical practice. *Postgrad Med J* [Epub ahead of print].

ARMSTRONG, M. J., HAZLEHURST, J. M., PARKER, R., KOUSHIAPPI, E., MANN, J., KHAN, S., PHILIPS, A., CHANDLER, L., JOHNSON, J., ROUND, R., HAYDON, G., KARAMAT, M., NEWSOME, P. N. & TOMLINSON, J. W. 2013c. Severe Asymptomatic Nonalcoholic Fatty Liver Disease in Routine Diabetes Care; a Multi-Disciplinary Team Approach to Diagnosis and Management. *QJM* [accepted, in press].

ARMSTRONG, M. J., HOULIHAN, D. D., BENTHAM, L., SHAW, J. C., CRAMB, R., OLLIFF, S., GILL, P. S., NEUBERGER, J. M., LILFORD, R. J. & NEWSOME, P. N. 2012. Presence and severity of non-alcoholic fatty liver disease in a large prospective primary care cohort. *J Hepatol*; 56: 234-40.

ARMSTRONG, M. J., HOULIHAN, D. D. & ROWE, I. A. 2010. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med*; 363: 1185; author reply 1186.

ARMSTRONG, M. J., HOULIHAN, D. D., ROWE, I. A., CLAUSEN, W. H. O., ELBRØND, B., GOUGH, S. C. L., TOMLINSON, J. W. & NEWSOME, P. N. 2013d. Safety and efficacy of liraglutide in patients with type 2 diabetes and elevated liver enzymes: individual patient data meta-analysis of the LEAD program. *Aliment Pharmacol Ther*; 37: 234-42.

ARODA, V. R., HENRY, R. R., HAN, J., HUANG, W., DEYOUNG, M. B., DARSOW, T. & HOOGWERF, B. J. 2012. Efficacy of GLP-1 receptor agonists and DPP-4 inhibitors: meta-analysis and systematic review. *Clin Ther*; 34: 1247-1258.e22.

ASCHA, M. S., HANOUNEH, I. A., LOPEZ, R., TAMIMI, T. A.-R., FELDSTEIN, A. F. & ZEIN, N. N. 2010. The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology*; 51: 1972-8.

ASTRUP, A., CARRARO, R., FINER, N., HARPER, A., KUNESOVA, M., LEAN, M. E. J., NISKANEN, L., RASMUSSEN, M. F., RISSANEN, A., RÖSSNER, S., SAVOLAINEN, M. J., VAN GAAL, L. & NN8022-1807-INVESTIGATORS. 2012. Safety, tolerability and sustained weight loss over 2 years with the once-daily human GLP-1 analog, liraglutide. *Int J Obes (Lond)*; 36: 843-54.

ASTRUP, A., RÖSSNER, S., VAN GAAL, L., RISSANEN, A., NISKANEN, L., AL HAKIM, M., MADSEN, J., RASMUSSEN, M. F., LEAN, M. E. J. & GROUP, N.-. 2009. Effects of liraglutide in

the treatment of obesity: a randomised, double-blind, placebo-controlled study. *Lancet*; 374: 1606-16.

AVILES-OLMOS, I., DICKSON, J., KEFALOPOULOU, Z., DJAMSHIDIAN, A., ELL, P., SODERLUND, T., WHITTON, P., WYSE, R., ISAACS, T., LEES, A., LIMOUSIN, P. & FOLTYNIE, T. 2013. Exenatide and the treatment of patients with Parkinson's disease. *J Clin Invest*; 123: 2730-6.

AVIV, V., MEIVAR-LEVY, I., RACHMUT, I. H., RUBINEK, T., MOR, E. & FERBER, S. 2009. Exendin-4 promotes liver cell proliferation and enhances the PDX-1-induced liver to pancreas transdifferentiation process. *J Biol Chem*; 284: 33509-20.

AYALA, J. E., BRACY, D. P., JAMES, F. D., BURMEISTER, M. A., WASSERMAN, D. H. & DRUCKER, D. J. 2010. Glucagon-like peptide-1 receptor knockout mice are protected from high-fat diet-induced insulin resistance. *Endocrinology*; 151: 4678-87.

BAGGIO, L. L. & DRUCKER, D. J. 2007. Biology of incretins: GLP-1 and GIP. *Gastroenterology*; 132: 2131-57.

BAN, K., NOYAN-ASHRAF, M. H., HOEFER, J., BOLZ, S.-S., DRUCKER, D. J. & HUSAIN, M. 2008. Cardioprotective and vasodilatory actions of glucagon-like peptide 1 receptor are mediated through both glucagon-like peptide 1 receptor-dependent and -independent pathways. *Circulation*; 117: 2340-50.

BARROWS, B. R. & PARKS, E. J. 2006. Contributions of different fatty acid sources to very low-density lipoprotein-triacylglycerol in the fasted and fed states. *J Clin Endocrinol Metab*; 91: 1446-52.

BEAVO, J. A. & BRUNTON, L. L. 2002. Cyclic nucleotide research -- still expanding after half a century. *Nat Rev Mol Cell Biol*; 3: 710-8.

BECHMANN, L. P., HANNIVOORT, R. A., GERKEN, G., HOTAMISLIGIL, G. S., TRAUNER, M. & CANBAY, A. 2012. The interaction of hepatic lipid and glucose metabolism in liver diseases. *J Hepatol*; 56: 952-64.

- BEDOJNI, G., BELLENTANI, S., MIGLIOLI, L., MASUTTI, F., PASSALACQUA, M., CASTIGLIONE, A. & TIRIBELLI, C. 2006. The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. *BMC Gastroenterol*; 6: 33.
- BEDOJNI, G., MIGLIOLI, L., MASUTTI, F., CASTIGLIONE, A., CROCÈ, L. S., TIRIBELLI, C. & BELLENTANI, S. 2007. Incidence and natural course of fatty liver in the general population: the Dionysos study. *Hepatology*; 46: 1387-91.
- BEDOJNI, G., MIGLIOLI, L., MASUTTI, F., TIRIBELLI, C., MARCHESINI, G. & BELLENTANI, S. 2005. Prevalence of and risk factors for nonalcoholic fatty liver disease: the Dionysos nutrition and liver study. *Hepatology*; 42: 44-52.
- BELFORT, R., HARRISON, S. A., BROWN, K., DARLAND, C., FINCH, J., HARDIES, J., BALAS, B., GASTALDELLI, A., TIO, F., PULCINI, J., BERRIA, R., MA, J. Z., DWIVEDI, S., HAVRANEK, R., FINCKE, C., DEFRONZO, R., BANNAYAN, G. A., SCHENKER, S. & CUSI, K. 2006. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. *N Engl J Med*; 355: 2297-2307.
- BELFORT, R., MANDARINO, L., KASHYAP, S., WIRFEL, K., PRATIPANAWATR, T., BERRIA, R., DEFRONZO, R. A. & CUSI, K. 2005. Dose-response effect of elevated plasma free fatty acid on insulin signaling. *Diabetes*; 54: 1640-8.
- BELL, G. I., SANCHEZ-PESCADOR, R., LAYBOURN, P. J. & NAJARIAN, R. C. 1983a. Exon duplication and divergence in the human preproglucagon gene. *Nature*; 304: 368-71.
- BELL, G. I., SANTERRE, R. F. & MULLENBACH, G. T. 1983b. Hamster preproglucagon contains the sequence of glucagon and two related peptides. *Nature*; 302: 716-8.
- BELLENTANI, S., BEDOGNI, G., MIGLIOLI, L. & TIRIBELLI, C. 2004. The epidemiology of fatty liver. *Eur J Gastroenterol Hepatol*; 16: 1087-93.
- BELLENTANI, S., SACCOCCIO, G., MASUTTI, F., CROCÈ, L. S., BRANDI, G., SASSO, F., CRISTANINI, G. & TIRIBELLI, C. 2000. Prevalence of and risk factors for hepatic steatosis in Northern Italy. *Ann Intern Med*; 132: 112-7.

- BELLENTANI, S., TIRIBELLI, C., SACCOCCIO, G., SODDE, M., FRATTI, N., DE MARTIN, C. & CRISTIANINI, G. 1994. Prevalence of chronic liver disease in the general population of northern Italy: the Dionysos Study. *Hepatology*; 20: 1442-9.
- BEN-SHLOMO, S., ZVIBEL, I., SHNELL, M., SHLOMAI, A., CHEPURKO, E., HALPERN, Z., BARZILAI, N., OREN, R. & FISHMAN, S. 2011. Glucagon-like peptide-1 reduces hepatic lipogenesis via activation of AMP-activated protein kinase. *J Hepatol*; 54: 1214-23.
- BERGENSTAL, R. M., WYSHAM, C., MACCONELL, L., MALLOY, J., WALSH, B., YAN, P., WILHELM, K., MALONE, J., PORTER, L. E. & GROUP, D.-S. 2010. Efficacy and safety of exenatide once weekly versus sitagliptin or pioglitazone as an adjunct to metformin for treatment of type 2 diabetes (DURATION-2): a randomised trial. *Lancet*; 376: 431-9.
- BERK, P. D. 2008. Regulatable fatty acid transport mechanisms are central to the pathophysiology of obesity, fatty liver, and metabolic syndrome. *Hepatology*; 48: 1362-76.
- BERTIN, E., ARNER, P., BOLINDER, J. & HAGSTRÖM-TOFT, E. 2001. Action of glucagon and glucagon-like peptide-1-(7-36) amide on lipolysis in human subcutaneous adipose tissue and skeletal muscle in vivo. *J Clin Endocrinol Metab*; 86: 1229-34.
- BEYMER, C., KOWDLEY, K. V., LARSON, A., EDMONSON, P., DELLINGER, E. P. & FLUM, D. R. 2003. Prevalence and predictors of asymptomatic liver disease in patients undergoing gastric bypass surgery. *Arch Surg*; 138: 1240-4.
- BHOGAL, R. H., HODSON, J., BARTLETT, D. C., WESTON, C. J., CURBISHLEY, S. M., HAUGHTON, E., WILLIAMS, K. T., REYNOLDS, G. M., NEWSOME, P. N., ADAMS, D. H. & AFFORD, S. C. 2011. Isolation of primary human hepatocytes from normal and diseased liver tissue: a one hundred liver experience. *PLoS ONE*; 6: e18222.
- BJERRE KNUDSEN, L., MADSEN, L. W., ANDERSEN, S., ALMHOLT, K., DE BOER, A. S., DRUCKER, D. J., GOTFREDSEN, C., EGEROD, F. L., HEGELUND, A. C., JACOBSEN, H., JACOBSEN, S. D., MOSES, A. C., MØLCK, A.-M., NIELSEN, H. S., NOWAK, J., SOLBERG, H., THI, T. D. L., ZDRAVKOVIC, M. & MOERCH, U. 2010. Glucagon-like Peptide-1 receptor agonists activate



rodent thyroid C-cells causing calcitonin release and C-cell proliferation. *Endocrinology*; 151: 1473-86.

BLACKMORE, P. F., MOJSOV, S., EXTON, J. H. & HABENER, J. F. 1991. Absence of insulinotropic glucagon-like peptide-I(7-37) receptors on isolated rat liver hepatocytes. *FEBS Lett*; 283: 7-10.

BLEVINS, T., PULLMAN, J., MALLOY, J., YAN, P., TAYLOR, K., SCHULTEIS, C., TRAUTMANN, M. & PORTER, L. 2011. DURATION-5: exenatide once weekly resulted in greater improvements in glycemic control compared with exenatide twice daily in patients with type 2 diabetes. *J Clin Endocrinol Metab*; 96: 1301-10.

BLOCK, G., WOODS, M., POTOSKY, A. & CLIFFORD, C. 1990. Validation of a self-administered diet history questionnaire using multiple diet records. *J Clin Epidemiol*; 43: 1327-1335.

BLONDE, L., KLEIN, E. J., HAN, J., ZHANG, B., MAC, S. M., POON, T. H., TAYLOR, K. L., TRAUTMANN, M. E., KIM, D. D. & KENDALL, D. M. 2006. Interim analysis of the effects of exenatide treatment on A1C, weight and cardiovascular risk factors over 82 weeks in 314 overweight patients with type 2 diabetes. *Diabetes Obes Metab*; 8: 436-47.

BLONDE, L. & RUSSELL-JONES, D. 2009. The safety and efficacy of liraglutide with or without oral antidiabetic drug therapy in type 2 diabetes: an overview of the LEAD 1-5 studies. *Diabetes Obes Metab*; 11 Suppl 3: 26-34.

BODEN, G. 2006. Fatty acid-induced inflammation and insulin resistance in skeletal muscle and liver. *Curr Diab Rep*; 6: 177-81.

BOHTE, A. E., VAN WERVEN, J. R., BIPAT, S. & STOKER, J. 2011. The diagnostic accuracy of US, CT, MRI and 1H-MRS for the evaluation of hepatic steatosis compared with liver biopsy: a meta-analysis. *Eur Radiol*; 21: 87-97.

BOSE, A. K., MOCANU, M. M., CARR, R. D., BRAND, C. L. & YELLON, D. M. 2005. Glucagon-like peptide 1 can directly protect the heart against ischemia/reperfusion injury. *Diabetes*; 54: 146-51.

- BOZA, C., RIQUELME, A., IBAÑEZ, L., DUARTE, I., NORERO, E., VIVIANI, P., SOZA, A., FERNANDEZ, J. I., RADDATZ, A., GUZMAN, S. & ARRESE, M. 2005. Predictors of nonalcoholic steatohepatitis (NASH) in obese patients undergoing gastric bypass. *Obes Surg*; 15: 1148-53.
- BRAVO, A. A., SHETH, S. G. & CHOPRA, S. 2001. Liver biopsy. *N Engl J Med*; 344: 495-500.
- BROWN, J. C., DRYBURGH, J. R., ROSS, S. A. & DUPRÉ, J. 1975. Identification and actions of gastric inhibitory polypeptide. *Recent Prog Horm Res*; 31: 487-532.
- BROWNING, J. D., SZCZEPANIAK, L. S., DOBBINS, R., NUREMBERG, P., HORTON, J. D., COHEN, J. C., GRUNDY, S. M. & HOBBS, H. H. 2004. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology*; 40: 1387-95.
- BRUBAKER, P. L. & DRUCKER, D. J. 2004. Minireview: Glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut, and central nervous system. *Endocrinology*; 145: 2653-9.
- BRUNT, E. M., JANNEY, C. G., DI BISCEGLIE, A. M., NEUSCHWANDER-TETRI, B. A. & BACON, B. R. 1999. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol*; 94: 2467-2474.
- BRUNT, E. M., KLEINER, D. E., WILSON, L. A., BELT, P., NEUSCHWANDER-TETRI, B. A. & (CRN), N. C. R. N. 2011. Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology*; 53: 810-20.
- BRUNT, E. M., KLEINER, D. E., WILSON, L. A., UNALP, A., BEHLING, C. E., LAVINE, J. E. & NEUSCHWANDER-TETRI, B. A. 2009. Portal chronic inflammation in nonalcoholic fatty liver disease (NAFLD): a histologic marker of advanced NAFLD-Clinicopathologic correlations from the nonalcoholic steatohepatitis clinical research network. *Hepatology*; 49: 809-820.
- BUGIANESI, E., GASTALDELLI, A., VANNI, E., GAMBINO, R., CASSADER, M., BALDI, S., PONTI, V., PAGANO, G., FERRANNINI, E. & RIZZETTO, M. 2005a. Insulin resistance in non-diabetic

patients with non-alcoholic fatty liver disease: sites and mechanisms. *Diabetologia*; 48: 634-42.

BUGIANESI, E., GENTILCORE, E., MANINI, R., NATALE, S., VANNI, E., VILLANOVA, N., DAVID, E., RIZZETTO, M. & MARCHESINI, G. 2005b. A randomized controlled trial of metformin versus vitamin E or prescriptive diet in nonalcoholic fatty liver disease. *Am J Gastroenterol*; 100: 1082-1090.

BUGIANESI, E., LEONE, N., VANNI, E., MARCHESINI, G., BRUNELLO, F., CARUCCI, P., MUSSO, A., DE PAOLIS, P., CAPUSSOTTI, L., SALIZZONI, M. & RIZZETTO, M. 2002. Expanding the natural history of nonalcoholic steatohepatitis: from cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology*; 123: 134-40.

BULLOCK, B. P., HELLER, R. S. & HABENER, J. F. 1996. Tissue distribution of messenger ribonucleic acid encoding the rat glucagon-like peptide-1 receptor. *Endocrinology*; 137: 2968-78.

BUSE, J. B., DRUCKER, D. J., TAYLOR, K. L., KIM, T., WALSH, B., HU, H., WILHELM, K., TRAUTMANN, M., SHEN, L. Z., PORTER, L. E. & GROUP, D.-S. 2010a. DURATION-1: exenatide once weekly produces sustained glycemic control and weight loss over 52 weeks. *Diabetes Care*; 33: 1255-61.

BUSE, J. B., GARBER, A., ROSENSTOCK, J., SCHMIDT, W. E., BRETT, J. H., VIDEBAEK, N., HOLST, J. & NAUCK, M. 2011. Liraglutide treatment is associated with a low frequency and magnitude of antibody formation with no apparent impact on glycemic response or increased frequency of adverse events: results from the Liraglutide Effect and Action in Diabetes (LEAD) trials. *J Clin Endocrinol Metab*; 96: 1695-702.

BUSE, J. B., HENRY, R. R., HAN, J., KIM, D. D., FINEMAN, M. S., BARON, A. D. & GROUP, E.-C. S. 2004. Effects of exenatide (exendin-4) on glycemic control over 30 weeks in sulfonylurea-treated patients with type 2 diabetes. *Diabetes Care*; 27: 2628-35.

BUSE, J. B., KLONOFF, D. C., NIELSEN, L. L., GUAN, X., BOWLUS, C. L., HOLCOMBE, J. H., MAGGS, D. G. & WINTLE, M. E. 2007. Metabolic effects of two years of exenatide treatment

on diabetes, obesity, and hepatic biomarkers in patients with type 2 diabetes: an interim analysis of data from the open-label, uncontrolled extension of three double-blind, placebo-controlled trials. *Clin Ther*; 29: 139-53.

BUSE, J. B., NAUCK, M., FORST, T., SHEU, W. H.-H., SHENOUDA, S. K., HEILMANN, C. R., HOOGWERF, B. J., GAO, A., BOARDMAN, M. K., FINEMAN, M., PORTER, L. & SCHERNTHANER, G. 2013. Exenatide once weekly versus liraglutide once daily in patients with type 2 diabetes (DURATION-6): a randomised, open-label study. *Lancet*; 381: 117-24.

BUSE, J. B., ROSENSTOCK, J., SESTI, G., SCHMIDT, W. E., MONTANYA, E., BRETT, J. H., ZYCHMA, M., BLONDE, L. & GROUP, L.-. 2009. Liraglutide once a day versus exenatide twice a day for type 2 diabetes: a 26-week randomised, parallel-group, multinational, open-label trial (LEAD-6). *Lancet*; 374: 39-47.

BUSE, J. B., SESTI, G., SCHMIDT, W. E., MONTANYA, E., CHANG, C.-T., XU, Y., BLONDE, L., ROSENSTOCK, J. & GROUP, L. 2010b. Switching to once-daily liraglutide from twice-daily exenatide further improves glycemic control in patients with type 2 diabetes using oral agents. *Diabetes Care*; 33: 1300-3.

BUTLER, A. E., CAMPBELL-THOMPSON, M., GURLO, T., DAWSON, D. W., ATKINSON, M. & BUTLER, P. C. 2013a. Marked expansion of exocrine and endocrine pancreas with incretin therapy in humans with increased exocrine pancreas dysplasia and the potential for glucagon-producing neuroendocrine tumors. *Diabetes*; 62: 2595-604.

BUTLER, P. C., ELASHOFF, M., ELASHOFF, R. & GALE, E. A. M. 2013b. A critical analysis of the clinical use of incretin-based therapies: Are the GLP-1 therapies safe? *Diabetes Care*; 36: 2118-25.

CABALLERÍA, L., AULADELL, M. A., TORÁN, P., MIRANDA, D., AZNAR, J., PERA, G., GIL, D., MUÑOZ, L., PLANAS, J., CANUT, S., BERNAD, J., AUBÀ, J., PIZARRO, G., AIZPURUA, M. M., ALTABA, A. & TIBAU, A. 2007. Prevalence and factors associated with the presence of non alcoholic fatty liver disease in an apparently healthy adult population in primary care units. *BMC Gastroenterol*; 7: 41.

- CALÈS, P., BOURSIER, J., OBERTI, F., GALLOIS, Y., ROUSSELET, M.-C., MOAL, V., MACCHI, L., CHEVAILLER, A. & LUNEL, F. 2009. [FibroMeters: a family of blood tests for liver fibrosis with high diagnostic performance and applicability in clinical practice]. *Pathol Biol*; 57: 459-62.
- CAMPOS, R. V., LEE, Y. C. & DRUCKER, D. J. 1994. Divergent tissue-specific and developmental expression of receptors for glucagon and glucagon-like peptide-1 in the mouse. *Endocrinology*; 134: 2156-64.
- CANADA Health. 2007. Standards for clinical trials in type 2 diabetes in Canada.
- CASTERA, L., FORNS, X. & ALBERTI, A. 2008. Non-invasive evaluation of liver fibrosis using transient elastography. *J Hepatol*; 48: 835-47.
- CHALASANI, N., YOUNOSSI, Z., LAVINE, J. E., DIEHL, A. M., BRUNT, E. M., CUSI, K., CHARLTON, M., SANYAL, A. J., ASSOCIATION, A. G., DISEASES, A. A. F. T. S. O. L. & GASTROENTEROLOGYH, A. C. O. 2012. The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. *Gastroenterology*; 142: 1592-609.
- CHALLA, T. D., BEATON, N., ARNOLD, M., RUDOFISKY, G., LANGHANS, W. & WOLFRUM, C. 2012. Regulation of adipocyte formation by GLP-1/GLP-1R signaling. *J Biol Chem*; 287: 6421-30.
- CHARLTON, M. R., BURNS, J. M., PEDERSEN, R. A., WATT, K. D., HEIMBACH, J. K. & DIERKHISING, R. A. 2011. Frequency and outcomes of liver transplantation for nonalcoholic steatohepatitis in the United States. *Gastroenterology*; 141: 1249-53.
- CHAVEZ-TAPIA, N. C., TELLEZ-AVILA, F. I., BARRIENTOS-GUTIERREZ, T., MENDEZ-SANCHEZ, N., LIZARDI-CERVERA, J. & URIBE, M. 2010. Bariatric surgery for non-alcoholic steatohepatitis in obese patients. *Cochrane Database Syst Rev*: CD007340.

- CHEN, H.-P., SHIEH, J.-J., CHANG, C.-C., CHEN, T.-T., LIN, J.-T., WU, M.-S., LIN, J.-H. & WU, C.-Y. 2013. Metformin decreases hepatocellular carcinoma risk in a dose-dependent manner: population-based and in vitro studies. *Gut*; 62: 606-15.
- CHIA, C. W. & EGAN, J. M. 2008. Incretin-based therapies in type 2 diabetes mellitus. *J Clin Endocrinol Metab*; 93: 3703-16.
- CHITTURI, S., ABEYGUNASEKERA, S., FARRELL, G. C., HOLMES-WALKER, J., HUI, J. M., FUNG, C., KARIM, R., LIN, R., SAMARASINGHE, D., LIDDLE, C., WELTMAN, M. & GEORGE, J. 2002. NASH and insulin resistance: Insulin hypersecretion and specific association with the insulin resistance syndrome. *Hepatology*; 35: 373-9.
- CHMP, E. M. A. 2013. Investigation into GLP-1 based diabetes therapies concluded [Press Release July 2013]. Available at: [http://www.ema.europa.eu/ema/index.jsp?curl=pages/news\\_and\\_events/news/2013/07/news\\_detail\\_001856.jsp&mid=WC0b01ac058004d5c1](http://www.ema.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2013/07/news_detail_001856.jsp&mid=WC0b01ac058004d5c1) [Accessed on 1/09/2013].
- CHO, Y. M., MERCHANT, C. E. & KIEFFER, T. J. 2012. Targeting the glucagon receptor family for diabetes and obesity therapy. *Pharmacol Ther*; 135: 247-78.
- CIGNARELLI, A., GIORGINO, F. & VETTOR, R. 2013. Pharmacologic agents for type 2 diabetes therapy and regulation of adipogenesis. *Arch Physiol Biochem*; [Epub ahead of print].
- CLARK, J. M., BRANCATI, F. L. & DIEHL, A. M. 2003. The prevalence and etiology of elevated aminotransferase levels in the United States. *Am J Gastroenterol*; 98: 960-7.
- COBBOLD, J., RAVEENDRAN, S. & PEAKE..., C. 2013. Piloting a multidisciplinary clinic for the management of non-alcoholic fatty liver disease: initial 5-year experience. *Frontline Gastroenterology*; [Epub ahead of print].
- COHEN, J. C., HORTON, J. D. & HOBBS, H. H. 2011. Human fatty liver disease: old questions and new insights. *Science*; 332: 1519-23.

COMBETTES, M. M. J. 2006. GLP-1 and type 2 diabetes: physiology and new clinical advances. *Curr Opin Pharmacol*; 6: 598-605.

CUNHA, D. A., LADRIÈRE, L., ORTIS, F., IGOILLO-ESTEVE, M., GURZOV, E. N., LUPI, R., MARCHETTI, P., EIZIRIK, D. L. & CNOP, M. 2009. Glucagon-like peptide-1 agonists protect pancreatic beta-cells from lipotoxic endoplasmic reticulum stress through upregulation of BiP and JunB. *Diabetes*; 58: 2851-62.

CUSI, K. 2012. Role of obesity and lipotoxicity in the development of nonalcoholic steatohepatitis: pathophysiology and clinical implications. *Gastroenterology*; 142: 711-725.e6.

CUSI, K., KASHYAP, S., GASTALDELLI, A., BAJAJ, M. & CERSOSIMO, E. 2007. Effects on insulin secretion and insulin action of a 48-h reduction of plasma free fatty acids with acipimox in nondiabetic subjects genetically predisposed to type 2 diabetes. *Am J Physiol Endocrinol Metab*; 292: E1775-81.

CUTHBERTSON, D. J., IRWIN, A., GARDNER, C. J., DAOUSI, C., PUREWAL, T., FURLONG, N., GOENKA, N., THOMAS, E. L., ADAMS, V. L., PUSHPAKOM, S. P., PIRMOHAMED, M. & KEMP, G. J. 2012. Improved Glycaemia Correlates with Liver Fat Reduction in Obese, Type 2 Diabetes, Patients Given Glucagon-Like Peptide-1 (GLP-1) Receptor Agonists. *PLoS ONE*; 7: e50117.

D'ALESSIO, D. A., KAHN, S. E., LEUSNER, C. R. & ENSINCK, J. W. 1994. Glucagon-like peptide 1 enhances glucose tolerance both by stimulation of insulin release and by increasing insulin-independent glucose disposal. *J Clin Invest*; 93: 2263-6.

DAM-LARSEN, S., FRANZMANN, M., ANDERSEN, I. B., CHRISTOFFERSEN, P., JENSEN, L. B., SØRENSEN, T. I. A., BECKER, U. & BENDTSEN, F. 2004. Long term prognosis of fatty liver: risk of chronic liver disease and death. *Gut*; 53: 750-5.

DANIEL, E. E., ANVARI, M., FOX-THRELKELD, J. E. T. & MCDONALD, T. J. 2002. Local, exendin-(9-39)-insensitive, site of action of GLP-1 in canine ileum. *Am J Physiol Gastrointest Liver Physiol*; 283: G595-602.

- DANIEL, S., BEN-MENACHEM, T., VASUDEVAN, G., MA, C. K. & BLUMENKEHL, M. 1999. Prospective evaluation of unexplained chronic liver transaminase abnormalities in asymptomatic and symptomatic patients. *Am J Gastroenterol*; 94: 3010-4.
- DAVIES, M. J., KELA, R. & KHUNTI, K. 2011. Liraglutide - overview of the preclinical and clinical data and its role in the treatment of type 2 diabetes. *Diabetes Obes Metab*; 13: 207-20.
- DAY, C. P. 2002. Pathogenesis of steatohepatitis. *Best Pract Res Clin Gastroenterol*; 16: 663-78.
- DAY, C. P. & JAMES, O. F. 1998. Steatohepatitis: a tale of two "hits"? *Gastroenterology*; 114: 842-5.
- DE RIDDER, R. J. J., SCHOON, E. J., SMULDERS, J. F., VAN HOUT, G. C. M., STOCKBRÜGGER, R. W. & KOEK, G. H. 2007. Review article: Non-alcoholic fatty liver disease in morbidly obese patients and the effect of bariatric surgery. *Aliment Pharmacol Ther*; 26 Suppl 2: 195-201.
- DE SILVA, A., SALEM, V., LONG, C. J., MAKWANA, A., NEWBOULD, R. D., RABINER, E. A., GHATEI, M. A., BLOOM, S. R., MATTHEWS, P. M., BEAVER, J. D. & DHILLO, W. S. 2011. The gut hormones PYY 3-36 and GLP-1 7-36 amide reduce food intake and modulate brain activity in appetite centers in humans. *Cell Metab*; 14: 700-6.
- DEACON, C. F., JOHNSEN, A. H. & HOLST, J. J. 1995. Degradation of glucagon-like peptide-1 by human plasma in vitro yields an N-terminally truncated peptide that is a major endogenous metabolite in vivo. *J Clin Endocrinol Metab*; 80: 952-7.
- DEFRONZO, R. A., RATNER, R. E., HAN, J., KIM, D. D., FINEMAN, M. S. & BARON, A. D. 2005. Effects of exenatide (exendin-4) on glycemic control and weight over 30 weeks in metformin-treated patients with type 2 diabetes. *Diabetes Care*; 28: 1092-100.
- DELGADO, E., LUQUE, M. A., ALCÁNTARA, A., TRAPOTE, M. A., CLEMENTE, F., GALERA, C., VALVERDE, I. & VILLANUEVA-PENACARRILLO, M. L. 1995. Glucagon-like peptide-1 binding to rat skeletal muscle. *Peptides*; 16: 225-9.



DENKER, P. S. & DIMARCO, P. E. 2006. Exenatide (exendin-4)-induced pancreatitis: a case report. *Diabetes Care*; 29: 471.

DIAMANT, M., VAN GAAL, L., STRANKS, S., NORTHRUP, J., CAO, D., TAYLOR, K. & TRAUTMANN, M. 2010. Once weekly exenatide compared with insulin glargine titrated to target in patients with type 2 diabetes (DURATION-3): an open-label randomised trial. *Lancet*; 375: 2234-43.

DIAO, L., LI, N., BRAYMAN, T. G., HOTZ, K. J. & LAI, Y. 2010. Regulation of MRP2/ABCC2 and BSEP/ABCB11 expression in sandwich cultured human and rat hepatocytes exposed to inflammatory cytokines TNF- $\alpha$ , IL-6, and IL-1 $\beta$ . *J Biol Chem*; 285: 31185-92.

DING, X., SAXENA, N. K., LIN, S., GUPTA, N. A., GUPTA, N. & ANANIA, F. A. 2006. Exendin-4, a glucagon-like protein-1 (GLP-1) receptor agonist, reverses hepatic steatosis in ob/ob mice. *Hepatology*; 43: 173-81.

DIRAISON, F., MOULIN, P. & BEYLOT, M. 2003. Contribution of hepatic de novo lipogenesis and reesterification of plasma non esterified fatty acids to plasma triglyceride synthesis during non-alcoholic fatty liver disease. *Diabetes Metab*; 29: 478-85.

DIRAISON, F., PACHIAUDI, C. & BEYLOT, M. 1996. In vivo measurement of plasma cholesterol and fatty acid synthesis with deuterated water: determination of the average number of deuterium atoms incorporated. *Metab Clin Exp*; 45: 817-21.

DIRAISON, F., PACHIAUDI, C. & BEYLOT, M. 1997. Measuring lipogenesis and cholesterol synthesis in humans with deuterated water: use of simple gas chromatographic/mass spectrometric techniques. *J Mass Spectrom*; 32: 81-6.

DONNELLY, K. L., SMITH, C. I., SCHWARZENBERG, S. J., JESSURUN, J., BOLDT, M. D. & PARKS, E. J. 2005. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest*; 115: 1343-51.

- DOWMAN, J. K., ARMSTRONG, M. J., TOMLINSON, J. W. & NEWSOME, P. N. 2011a. Current therapeutic strategies in non-alcoholic fatty liver disease. *Diabetes Obes Metab*; 13: 692-702.
- DOWMAN, J. K., TOMLINSON, J. W. & NEWSOME, P. N. 2010. Pathogenesis of non-alcoholic fatty liver disease. *QJM*; 103: 71-83.
- DOWMAN, J. K., TOMLINSON, J. W. & NEWSOME, P. N. 2011b. Systematic review: the diagnosis and staging of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. *Aliment Pharmacol Ther*; 33: 525-40.
- DRUCKER, D. J. 2006. The biology of incretin hormones. *Cell Metab*; 3: 153-65.
- DUFOUR, J. F., ONETA, C. M., GONVERS, J. J., BIHL, F., CERNY, A., CEREDA, J. M., ZALA, J. F., HELBLING, B., STEUERWALD, M. & ZIMMERMANN, A. 2006. Randomized placebo-controlled trial of ursodeoxycholic acid with vitamin e in nonalcoholic steatohepatitis. *Clin Gastroenterol Hepatol*; 4: 1537-1543.
- DUNPHY, J. L., TAYLOR, R. G. & FULLER, P. J. 1998. Tissue distribution of rat glucagon receptor and GLP-1 receptor gene expression. *Mol Cell Endocrinol*; 141: 179-86.
- DUPRE, J., ROSS, S. A., WATSON, D. & BROWN, J. C. 1973. Stimulation of insulin secretion by gastric inhibitory polypeptide in man. *J Clin Endocrinol Metab*; 37: 826-8.
- EDWARDS, C. M., EDWARDS, A. V. & BLOOM, S. R. 1997. Cardiovascular and pancreatic endocrine responses to glucagon-like peptide-1(7-36) amide in the conscious calf. *Exp Physiol*; 82: 709-16.
- EGAN, J. M., CLOCQUET, A. R. & ELAHI, D. 2002. The insulinotropic effect of acute exendin-4 administered to humans: comparison of nondiabetic state to type 2 diabetes. *J Clin Endocrinol Metab*; 87: 1282-1290.
- EGAN, J. M., MONTROSE-RAFIZADEH, C., WANG, Y., BERNIER, M. & ROTH, J. 1994. Glucagon-like peptide-1(7-36) amide (GLP-1) enhances insulin-stimulated glucose metabolism in 3T3-

L1 adipocytes: one of several potential extrapancreatic sites of GLP-1 action. *Endocrinology*; 135: 2070-5.

EGUCHI, Y., EGUCHI, T., MIZUTA, T., IDE, Y., YASUTAKE, T., IWAKIRI, R., HISATOMI, A., OZAKI, I., YAMAMOTO, K., KITAJIMA, Y., KAWAGUCHI, Y., KUROKI, S. & ONO, N. 2006. Visceral fat accumulation and insulin resistance are important factors in nonalcoholic fatty liver disease. *J Gastroenterol*; 41: 462-9.

EKSTEDT, M., FRANZÉN, L. E., MATHIESEN, U. L., THORELIUS, L., HOLMQVIST, M., BODEMAR, G. & KECHAGIAS, S. 2006. Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology*; 44: 865-73.

EL-SERAG, H. B., TRAN, T. & EVERHART, J. E. 2004. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. *Gastroenterology*; 126: 460-8.

ELASHOFF, M., MATVEYENKO, A. V., GIER, B., ELASHOFF, R. & BUTLER, P. C. 2011. Pancreatitis, pancreatic, and thyroid cancer with glucagon-like peptide-1-based therapies. *Gastroenterology*; 141: 150-6.

ELI LILY AND COMPANY LTD. 2011a. *SmPc Exenatide ER (Bydureon®)* [Online]. Available at: [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/EPAR\\_-\\_Product\\_Information/human/002020/WC500108241.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/002020/WC500108241.pdf) [Accessed 20/08/2013].

ELI LILY AND COMPANY LTD. 2011b. *SmPc for Exenatide (Byetta®)* [Online]. Available at: [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/EPAR\\_-\\_Product\\_Information/human/000698/WC500051845.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000698/WC500051845.pdf) [Accessed 20/08/2013].

ELLIOTT, R. M., MORGAN, L. M., TREDGER, J. A., DEACON, S., WRIGHT, J. & MARKS, V. 1993. Glucagon-like peptide-1 (7-36)amide and glucose-dependent insulintropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns. *J Endocrinol*; 138: 159-66.

ELLRICHMANN, M., VOLLMER, K., SCHRADER, H., BANASCH, M., SCHMIDT, W. E. & MEIER, J. 2009. Sustained virological response during exenatide treatment in a patient with hepatitis C and nonalcoholic steatohepatitis. *Am J Gastroenterol*; 104: 3112-3114.

ELRICK, H., STIMMLER, L., HLAD, C. J. & ARAI, Y. 1964. Plasma insulin response to oral and intravenous glucose administration. *J Clin Endocrinol Metab*; 24: 1076-82.

ENG, J., KLEINMAN, W. A., SINGH, L., SINGH, G. & RAUFMAN, J. P. 1992. Isolation and characterization of exendin-4, an exendin-3 analogue, from *Heloderma suspectum* venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. *J Biol Chem*; 267: 7402-5.

EUROPEAN MEDICINES AGENCY. 2009. European public assessment report (EPAR) for Victoza. EMA/Committee for Medicinal Products for Human Use. Available at [http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/001026/human\\_med\\_001137](http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/001026/human_med_001137) [Accessed 28/08/2013].

EUROPEAN MEDICINES AGENCY, 2012. Summary of Product Characteristics (SmPc) for Victoza. Available at [:http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/001026/human\\_med\\_001137](http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/001026/human_med_001137) [Accessed 20/08/2013].

FABBRINI, E., MOHAMMED, B. S., MAGKOS, F., KORENBLAT, K. M., PATTERSON, B. W. & KLEIN, S. 2008. Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. *Gastroenterology*; 134: 424-31.

FELDSTEIN, A. E., CHARATCHAROENWITTHAYA, P., TREEPRASERTSUK, S., BENSON, J. T., ENDERS, F. B. & ANGULO, P. 2009a. The natural history of non-alcoholic fatty liver disease in children: a follow-up study for up to 20 years. *Gut*; 58: 1538-44.

FELDSTEIN, A. E., WIECKOWSKA, A., LOPEZ, A. R., LIU, Y. C., ZEIN, N. N. & MCCULLOUGH, A. J. 2009b. Cytokeratin-18 fragment levels as noninvasive biomarkers for nonalcoholic steatohepatitis: a multicenter validation study. *Hepatology*; 50: 1072-1078.

FERREIRA, I., HENRY, R. M. A., TWISK, J. W. R., VAN MECHELEN, W., KEMPER, H. C. G., STEHOUWER, C. D. A. & STUDY, A. G. A. H. L. 2005. The metabolic syndrome, cardiopulmonary fitness, and subcutaneous trunk fat as independent determinants of arterial stiffness: the Amsterdam Growth and Health Longitudinal Study. *Arch Intern Med*; 165: 875-82.

FINEGOOD, D. T., BERGMAN, R. N. & VRANIC, M. 1987. Estimation of endogenous glucose production during hyperinsulinemic-euglycemic glucose clamps. Comparison of unlabeled and labeled exogenous glucose infusates. *Diabetes*; 36: 914-24.

FLOCK, G., BAGGIO, L. L., LONGUET, C. & DRUCKER, D. J. 2007. Incretin receptors for glucagon-like peptide 1 and glucose-dependent insulintropic polypeptide are essential for the sustained metabolic actions of vildagliptin in mice. *Diabetes*; 56: 3006-13.

FOLCH, J., LEES, M. & SLOANE STANLEY, G. H. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem*; 226: 497-509.

FONTANA, L., EAGON, J. C., TRUJILLO, M. E., SCHERER, P. E. & KLEIN, S. 2007. Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. *Diabetes*; 56: 1010-3.

FRACANZANI, A. L., VALENTI, L., BUGIANESI, E., ANDREOLETTI, M., COLLI, A., VANNI, E., BERTELLI, C., FATTA, E., BIGNAMINI, D., MARCHESINI, G. & FARGION, S. 2008. Risk of severe liver disease in nonalcoholic fatty liver disease with normal aminotransferase levels: a role for insulin resistance and diabetes. *Hepatology*; 48: 792-8.

FRANKS, A. S., LEE, P. H. & GEORGE, C. M. 2012. Pancreatitis: a potential complication of liraglutide? *Ann Pharmacother*; 46: 1547-53.

FRIEDRICH-RUST, M., ONG, M.-F., MARTENS, S., SARRAZIN, C., BOJUNGA, J., ZEUZEM, S. & HERRMANN, E. 2008. Performance of transient elastography for the staging of liver fibrosis: a meta-analysis. *Gastroenterology*; 134: 960-74.

- FÜRNSINN, C., EBNER, K. & WALDHÄUSL, W. 1995. Failure of GLP-1(7-36)amide to affect glycogenesis in rat skeletal muscle. *Diabetologia*; 38: 864-7.
- GAO, H., WANG, X., ZHANG, Z., YANG, Y., YANG, J., LI, X. & NING, G. 2007. GLP-1 amplifies insulin signaling by up-regulation of IRbeta, IRS-1 and Glut4 in 3T3-L1 adipocytes. *Endocrine*; 32: 90-5.
- GARBER, A., HENRY, R., RATNER, R., GARCIA-HERNANDEZ, P. A., RODRIGUEZ-PATTZI, H., OLVERA-ALVAREZ, I., HALE, P. M., ZDRAVKOVIC, M., BODE, B. & GROUP, L.-. 2009. Liraglutide versus glimepiride monotherapy for type 2 diabetes (LEAD-3 Mono): a randomised, 52-week, phase III, double-blind, parallel-treatment trial. *Lancet*; 373: 473-81.
- GARBER, A., HENRY, R. R., RATNER, R., HALE, P., CHANG, C. T., BODE, B. & GROUP, L.-. 2011. Liraglutide, a once-daily human glucagon-like peptide 1 analogue, provides sustained improvements in glycaemic control and weight for 2 years as monotherapy compared with glimepiride in patients with type 2 diabetes. *Diabetes Obes Metab*; 13: 348-56.
- GARG, A. 2004. Regional adiposity and insulin resistance. *J Clin Endocrinol Metab*; 89: 4206-10.
- GARG, R., CHEN, W. & PENDERGRASS, M. 2010. Acute pancreatitis in type 2 diabetes treated with exenatide or sitagliptin: a retrospective observational pharmacy claims analysis. *Diabetes Care*; 33: 2349-54.
- GASTALDELLI, A., CUSI, K., PETTITI, M., HARDIES, J., MIYAZAKI, Y., BERRIA, R., BUZZIGOLI, E., SIRONI, A. M., CERSOSIMO, E., FERRANNINI, E. & DEFRONZO, R. A. 2007. Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. *Gastroenterology*; 133: 496-506.
- GASTALDELLI, A., HARRISON, S., BELFORT-AGUIAR, R., HARDIES, J., BALAS, B., SCHENKER, S. & CUSI, K. 2010. Pioglitazone in the treatment of NASH: the role of adiponectin. *Aliment Pharmacol Ther*; 32: 769-75.

- GASTALDELLI, A., HARRISON, S. A., BELFORT-AGUILAR, R., HARDIES, L. J., BALAS, B., SCHENKER, S. & CUSI, K. 2009. Importance of changes in adipose tissue insulin resistance to histological response during thiazolidinedione treatment of patients with nonalcoholic steatohepatitis. *Hepatology*; 50: 1087-93.
- GATHERCOLE, L. L., MORGAN, S. A., BUJALSKA, I. J., HAUTON, D., STEWART, P. M. & TOMLINSON, J. W. 2011. Regulation of lipogenesis by glucocorticoids and insulin in human adipose tissue. *PLoS ONE*; 6: e26223.
- GEDULIN, B. R., NIKOULINA, S. E., SMITH, P. A., GEDULIN, G., NIELSEN, L. L., BARON, A. D., PARKES, D. G. & YOUNG, A. A. 2005. Exenatide (exendin-4) improves insulin sensitivity and  $\beta$ -cell mass in insulin-resistant obese fa/fa Zucker rats independent of glycemia and body weight. *Endocrinology*; 146: 2069-76.
- GEJL, M., EGEFJORD, L., LERCHE, S., VANG, K., BIBBY, B. M., HOLST, J. J., MENGEL, A., MØLLER, N., RUNGBY, J., BROCK, B. & GJEDDE, A. 2012. Glucagon-like peptide-1 decreases intracerebral glucose content by activating hexokinase and changing glucose clearance during hyperglycemia. *J Cereb Blood Flow Metab*; 32: 2146-52.
- GEORGESCU, E. F., IONESCU, R., NICULESCU, M., MOGOANTA, L. & VANCICA, L. 2009. Angiotensin-receptor blockers as therapy for mild-to-moderate hypertension-associated non-alcoholic steatohepatitis. *World J Gastroenterol*; 15: 942-54.
- GHIGLIONE, M., BLAZQUEZ, E., UTTENTHAL, L. O., DE DIEGO, J. G., ALVAREZ, E., GEORGE, S. K. & BLOOM, S. R. 1985. Glucagon-like peptide-1 does not have a role in hepatic carbohydrate metabolism. *Diabetologia*; 28: 920-1.
- GHOLAM, P. M., FLANCBAUM, L., MACHAN, J. T., CHARNEY, D. A. & KOTLER, D. P. 2007. Nonalcoholic fatty liver disease in severely obese subjects. *Am J Gastroenterol*; 102: 399-408.
- GIER, B., MATVEYENKO, A. V., KIRAKOSSIAN, D., DAWSON, D., DRY, S. M. & BUTLER, P. C. 2012. Chronic GLP-1 receptor activation by exendin-4 induces expansion of pancreatic duct

glands in rats and accelerates formation of dysplastic lesions and chronic pancreatitis in the Kras(G12D) mouse model. *Diabetes*; 61: 1250-62.

GÖKE, R., FEHMANN, H. C., LINN, T., SCHMIDT, H., KRAUSE, M., ENG, J. & GÖKE, B. 1993. Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells. *J Biol Chem*; 268: 19650-5.

GONZÁLEZ, N., ACITORES, A., SANCHO, V., VALVERDE, I. & VILLANUEVA-PEÑACARRILLO, M. L. 2005. Effect of GLP-1 on glucose transport and its cell signalling in human myocytes. *Regul Pept*; 126: 203-11.

GONZALEZ-PEREZ, A., SCHLIENGER, R. G. & RODRÍGUEZ, L. A. G. 2010. Acute pancreatitis in association with type 2 diabetes and antidiabetic drugs: a population-based cohort study. *Diabetes Care*; 33: 2580-5.

GOODPASTER, B. H., THAETE, F. L., SIMONEAU, J. A. & KELLEY, D. E. 1997. Subcutaneous abdominal fat and thigh muscle composition predict insulin sensitivity independently of visceral fat. *Diabetes*; 46: 1579-85.

GOUGH, S. C. L. 2012. Liraglutide: from clinical trials to clinical practice. *Diabetes Obes Metab*; 14 Suppl 2: 33-40.

GRATTAGLIANO, I., DE BARI, O., BERNARDO, T. C., OLIVEIRA, P. J., WANG, D. Q.-H. & PORTINCASA, P. 2012. Role of mitochondria in nonalcoholic fatty liver disease--from origin to propagation. *Clin Biochem*; 45: 610-8.

GRIGORESCU, M., CRISAN, D., RADU, C., GRIGORESCU, M. D., SPARCHEZ, Z. & SERBAN, A. 2012. A novel pathophysiological-based panel of biomarkers for the diagnosis of nonalcoholic steatohepatitis. *J Physiol Pharmacol*; 63: 347-53.

GRIMM, M., HAN, J., WEAVER, C., GRIFFIN, P., SCHULTEIS, C. T., DONG, H. & MALLOY, J. 2013. Efficacy, safety, and tolerability of exenatide once weekly in patients with type 2 diabetes mellitus: an integrated analysis of the DURATION trials. *Postgrad Med*; 125: 47-57.



GU, W., LLOYD, D. J., CHINOOKSWONG, N., KOMOROWSKI, R., SIVITS, G., GRAHAM, M., WINTERS, K. A., YAN, H., BOROS, L. G., LINDBERG, R. A. & VÉNIANT, M. M. 2011. Pharmacological targeting of glucagon and glucagon-like peptide 1 receptors has different effects on energy state and glucose homeostasis in diet-induced obese mice. *J Pharmacol Exp Ther*; 338: 70-81.

GUHA, I. N., PARKES, J., RODERICK, P., CHATTOPADHYAY, D., CROSS, R., HARRIS, S., KAYE, P., BURT, A. D., RYDER, S. D., AITHAL, G. P., DAY, C. P. & ROSENBERG, W. M. 2008. Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: Validating the European Liver Fibrosis Panel and exploring simple markers. *Hepatology*; 47: 455-60.

GUHA, I. N., PARKES, J., RODERICK, P. R., HARRIS, S. & ROSENBERG, W. M. 2006. Non-invasive markers associated with liver fibrosis in non-alcoholic fatty liver disease. *Gut*; 55: 1650-60.

GUPTA, N. A., MELLS, J., DUNHAM, R. M., GRAKOU, A., HANDY, J., SAXENA, N. K. & ANANIA, F. A. 2010. Glucagon-like peptide-1 receptor is present on human hepatocytes and has a direct role in decreasing hepatic steatosis in vitro by modulating elements of the insulin signaling pathway. *Hepatology*; 51: 1584-92.

GUTNIAK, M., ORSKOV, C., HOLST, J. J., AHRÉN, B. & EFENDIC, S. 1992. Antidiabetogenic effect of glucagon-like peptide-1 (7-36)amide in normal subjects and patients with diabetes mellitus. *N Engl J Med*; 326: 1316-22.

GUTZWILLER, J.-P., TSCHOPP, S., BOCK, A., ZEHNDER, C. E., HUBER, A. R., KREYENBUEHL, M., GUTMANN, H., DREWE, J., HENZEN, C., GOEKE, B. & BEGLINGER, C. 2004. Glucagon-like peptide 1 induces natriuresis in healthy subjects and in insulin-resistant obese men. *J Clin Endocrinol Metab*; 89: 3055-61.

HAMAGUCHI, M., KOJIMA, T., TAKEDA, N., NAKAGAWA, T., TANIGUCHI, H., FUJII, K., OMATSU, T., NAKAJIMA, T., SARUI, H., SHIMAZAKI, M., KATO, T., OKUDA, J. & IDA, K. 2005. The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. *Ann Intern Med*; 143: 722-8.

- HANSEN, L., DEACON, C. F., ORSKOV, C. & HOLST, J. J. 1999. Glucagon-like peptide-1-(7-36)amide is transformed to glucagon-like peptide-1-(9-36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. *Endocrinology*; 140: 5356-5363.
- HARRISON, S. A., FECHT, W., BRUNT, E. M. & NEUSCHWANDER-TETRI, B. A. 2009. Orlistat for overweight subjects with nonalcoholic steatohepatitis: A randomized, prospective trial. *Hepatology*; 49: 80-86.
- HARRISON, S. A., OLIVER, D., ARNOLD, H. L., GOGIA, S. & NEUSCHWANDER-TETRI, B. A. 2008. Development and validation of a simple NAFLD clinical scoring system for identifying patients without advanced disease. *Gut*; 57: 1441-7.
- HARRISON, S. A., TORGERSON, S., HAYASHI, P., WARD, J. & SCHENKER, S. 2003. Vitamin E and vitamin C treatment improves fibrosis in patients with nonalcoholic steatohepatitis. *Am J Gastroenterol*; 98: 2485-90.
- HAUKELAND, J. W., KONOPSKI, Z., EGGESBØ, H. B., VON VOLKMANN, H. L., RASCHPICHLER, G., BJØRO, K., HAALAND, T., LØBERG, E. M. & BIRKELAND, K. 2009. Metformin in patients with non-alcoholic fatty liver disease: a randomized, controlled trial. *Scand J Gastroenterol*; 44: 853-60.
- HAZLEHURST, J. M., GATHERCOLE, L. L., NASIRI, M., ARMSTRONG, M. J., BORROWS, S., YU, J., WAGENMAKERS, A. J. M., STEWART, P. M. & TOMLINSON, J. W. 2013. Glucocorticoids fail to cause insulin resistance in human subcutaneous adipose tissue in vivo. *J Clin Endocrinol Metab*; 98: 1631-40.
- HAZLEHURST, J. M. & TOMLINSON, J. W. 2013. Mechanisms in Endocrinology: Non-alcoholic fatty liver disease in common endocrine disorders. *Eur J Endocrinol*; 169:R27-37.
- HEINE, R. J., VAN GAAL, L. F., JOHNS, D., MIHM, M. J., WIDEL, M. H., BRODOWS, R. G. & GROUP, G. S. 2005. Exenatide versus insulin glargine in patients with suboptimally controlled type 2 diabetes: a randomized trial. *Ann Intern Med*; 143: 559-69.

HERMANSEN, K., BAEKDAL, T. A., DÜRING, M., PIETRASZEK, A., MORTENSEN, L. S., JØRGENSEN, H. & FLINT, A. 2013. Liraglutide suppresses postprandial triglyceride and apolipoprotein B48 elevations after a fat-rich meal in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled, cross-over trial. *Diabetes Obes Metab*; [Epub ahead of print].

HERRMANN-RINKE, C., VÖGE, A., HESS, M. & GÖKE, B. 1995. Regulation of glucagon-like peptide-1 secretion from rat ileum by neurotransmitters and peptides. *J Endocrinol*; 147: 25-31.

HIMENO, T., KAMIYA, H., NARUSE, K., HARADA, N., OZAKI, N., SEINO, Y., SHIBATA, T., KONDO, M., KATO, J., OKAWA, T., FUKAMI, A., HAMADA, Y., INAGAKI, N., SEINO, Y., DRUCKER, D. J., OISO, Y. & NAKAMURA, J. 2011. Beneficial effects of exendin-4 on experimental polyneuropathy in diabetic mice. *Diabetes*; 60: 2397-406.

HOLST, J. J. 2007. The physiology of glucagon-like peptide 1. *Physiol Rev*; 87: 1409-1439.

HOU, X., XU, S., MAITLAND-TOOLAN, K. A., SATO, K., JIANG, B., IDO, Y., LAN, F., WALSH, K., WIERZBICKI, M., VERBEUREN, T. J., COHEN, R. A. & ZANG, M. 2008. SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *J Biol Chem*; 283: 20015-26.

HÜBSCHER, S. G. 2006. Histological assessment of non-alcoholic fatty liver disease. *Histopathology*; 49: 450-65.

HULTCRANTZ, R., GLAUMANN, H., LINDBERG, G. & NILSSON, L. H. 1986. Liver investigation in 149 asymptomatic patients with moderately elevated activities of serum aminotransferases. *Scand J Gastroenterol*; 21: 109-13.

HUMPHREYS, E. H., WILLIAMS, K. T., ADAMS, D. H. & AFFORD, S. C. 2010. Primary and malignant cholangiocytes undergo CD40 mediated Fas dependent apoptosis, but are insensitive to direct activation with exogenous Fas ligand. *PLoS ONE*; 5: e14037.

- IDILMAN, R., MIZRAK, D., CORAPCIOGLU, D., BEKTAS, M., DOGANAY, B., SAYKI, M., COBAN, S., ERDEN, E., SOYKAN, I., EMRAL, R., UYSAL, A. R. & OZDEN, A. 2008. Clinical trial: insulin-sensitizing agents may reduce consequences of insulin resistance in individuals with non-alcoholic steatohepatitis. *Aliment Pharmacol Ther*; 28: 200-208.
- IDRIS, I., PATIAG, D., GRAY, S. & DONNELLY, R. 2002. Exendin-4 increases insulin sensitivity via a PI-3-kinase-dependent mechanism: contrasting effects of GLP-1. *Biochem Pharmacol*; 63: 993-6.
- ISHAK, K., BAPTISTA, A., BIANCHI, L., CALLEA, F., DE GROOTE, J., GUDAT, F., DENK, H., DESMET, V., KORB, G. & MACSWEEN, R. N. 1995. Histological grading and staging of chronic hepatitis. *J Hepatol*; 22: 696-699.
- JAMDAR, S. C. 1978. Glycerolipid biosynthesis in rat adipose tissue. Influence of adipose-cell size and site of adipose tissue on triacylglycerol formation in lean and obese rats. *Biochem J*; 170: 153-60.
- JENDLE, J., NAUCK, M. A., MATTHEWS, D. R., FRID, A., HERMANSEN, K., DÜRING, M., ZDRAVKOVIC, M., STRAUSS, B. J., GARBER, A. J. & GROUPS, L.-. 2009. Weight loss with liraglutide, a once-daily human glucagon-like peptide-1 analogue for type 2 diabetes treatment as monotherapy or added to metformin, is primarily as a result of a reduction in fat tissue. *Diabetes Obes Metab*; 11: 1163-72.
- JOKA, D., WAHL, K., MOELLER, S., SCHLUE, J., VASKE, B., BAHR, M. J., MANNS, M. P., SCHULZE-OSTHOFF, K. & BANTEI, H. 2012. Prospective biopsy-controlled evaluation of cell death biomarkers for prediction of liver fibrosis and nonalcoholic steatohepatitis. *Hepatology*; 55: 455-64.
- JOPLIN, R., STRAIN, A. J. & NEUBERGER, J. M. 1989. Immuno-isolation and culture of biliary epithelial cells from normal human liver. *In Vitro Cell Dev Biol*; 25: 1189-92.
- JOU, J., CHOI, S. S. & DIEHL, A. M. 2008. Mechanisms of disease progression in nonalcoholic fatty liver disease. *Semin Liver Dis*; 28: 370-9.

- KAMENETSKY, M., MIDDELHAUFE, S., BANK, E. M., LEVIN, L. R., BUCK, J. & STEEGBORN, C. 2006. Molecular details of cAMP generation in mammalian cells: a tale of two systems. *J Mol Biol*; 362: 623-39.
- KARPE, F., DICKMANN, J. R. & FRAYN, K. N. 2011. Fatty acids, obesity, and insulin resistance: time for a reevaluation. *Diabetes*; 60: 2441-9.
- KASHYAP, S., BELFORT, R., GASTALDELLI, A., PRATIPANAWATR, T., BERRIA, R., PRATIPANAWATR, W., BAJAJ, M., MANDARINO, L., DEFRONZO, R. & CUSI, K. 2003. A sustained increase in plasma free fatty acids impairs insulin secretion in nondiabetic subjects genetically predisposed to develop type 2 diabetes. *Diabetes*; 52: 2461-74.
- KENDALL, D. M., RIDDLE, M. C., ROSENSTOCK, J., ZHUANG, D., KIM, D. D., FINEMAN, M. S. & BARON, A. D. 2005. Effects of exenatide (exendin-4) on glycemic control over 30 weeks in patients with type 2 diabetes treated with metformin and a sulfonylurea. *Diabetes Care*; 28: 1083-91.
- KENNY, P. R., BRADY, D. E., TORRES, D. M., RAGOZZINO, L., CHALASANI, N. & HARRISON, S. A. 2010. Exenatide in the treatment of diabetic patients with non-alcoholic steatohepatitis: a case series. *Am J Gastroenterol*; 105: 2707-9.
- KERN, P. A., RANGANATHAN, S., LI, C., WOOD, L. & RANGANATHAN, G. 2001. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab*; 280: E745-51.
- KIM, D., KIM, W. R., KIM, H. J. & THERNEAU, T. M. 2013a. Association between noninvasive fibrosis markers and mortality among adults with nonalcoholic fatty liver disease in the United States. *Hepatology*; 57: 1357-65.
- KIM, D., KIM, W. R., TALWALKAR, J. A., KIM, H. J. & EHMAN, R. L. 2013b. Advanced fibrosis in nonalcoholic fatty liver disease: noninvasive assessment with MR elastography. *Radiology*; 268: 411-9.

KLEIN, S., FONTANA, L., YOUNG, L., COGGAN, A.R., KILO, C., PATTERSON, B.W. & MOHAMMED, S. 2004. Absence of an effect of liposuction on insulin action and risk factors for coronary heart disease. *NEJM*; 350: 2549-57.

KLEINER, D. E., BRUNT, E. M., VAN NATTA, M., BEHLING, C., CONTOS, M. J., CUMMINGS, O. W., FERRELL, L. D., LIU, Y.-C., TORBENSON, M. S., UNALP-ARIDA, A., YEH, M., MCCULLOUGH, A. J., SANYAL, A. J. & NETWORK, N. 2005. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*; 41: 1313-21.

KLER, R. S., SHERRATT, H. S. & TURNBULL, D. M. 1992. The measurement of mitochondrial beta-oxidation by release of  $3H_2O$  from [9,10- $3H$ ]hexadecanoate: application to skeletal muscle and the use of inhibitors as models of metabolic disease. *Biochem Med Metab Biol*; 47: 145-56.

KLONOFF, D. C., BUSE, J. B., NIELSEN, L. L., GUAN, X., BOWLUS, C. L., HOLCOMBE, J. H., WINTLE, M. E. & MAGGS, D. G. 2008. Exenatide effects on diabetes, obesity, cardiovascular risk factors and hepatic biomarkers in patients with type 2 diabetes treated for at least 3 years. *Curr Med Res Opin*; 24: 275-86.

KNUDSEN, L. B. 2010. Liraglutide: the therapeutic promise from animal models. *Int J Clin Pract Suppl*; 64: 4-11.

KNUDSEN, L. B., NIELSEN, P. F., HUUSFELDT, P. O., JOHANSEN, N. L., MADSEN, K., PEDERSEN, F. Z., THØGERSEN, H., WILKEN, M. & AGERSØ, H. 2000. Potent derivatives of glucagon-like peptide-1 with pharmacokinetic properties suitable for once daily administration. *J Med Chem*; 43: 1664-9.

KÖRNER, M., STÖCKLI, M., WASER, B. & REUBI, J. C. 2007. GLP-1 receptor expression in human tumors and human normal tissues: potential for in vivo targeting. *J Nucl Med*; 48: 736-43.

KOSKA, J. 2012. Incretins and preservation of endothelial function. *Cardiovasc Hematol Agents Med Chem*; 10: 295-308.

- KOTRONEN, A., PELTONEN, M., HAKKARAINEN, A., SEVASTIANOVA, K., BERGHOLM, R., JOHANSSON, L. M., LUNDBOM, N., RISSANEN, A., RIDDERSTRÅLE, M., GROOP, L., ORHO-MELANDER, M. & YKI-JÄRVINEN, H. 2009. Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. *Gastroenterology*; 137: 865-72.
- KOTRONEN, A., SEPPÄNEN-LAAKSO, T., WESTERBACKA, J., KIVILUOTO, T., AROLA, J., RUSKEEPÄÄ, A.-L., YKI-JÄRVINEN, H. & ORESIC, M. 2010. Comparison of lipid and fatty acid composition of the liver, subcutaneous and intra-abdominal adipose tissue, and serum. *Obesity (Silver Spring)*; 18: 937-44.
- KOWDLEY, K. V. & CALDWELL, S. 2006. Nonalcoholic steatohepatitis: a twenty-first century epidemic? *J Clin Gastroenterol*; 40: S2-S4.
- KREYMANN, B., WILLIAMS, G., GHATEI, M. A. & BLOOM, S. R. 1987. Glucagon-like peptide-1 7-36: a physiological incretin in man. *Lancet*; 2: 1300-4.
- LALOR, P. F., EDWARDS, S., MCNAB, G., SALMI, M., JALKANEN, S. & ADAMS, D. H. 2002. Vascular adhesion protein-1 mediates adhesion and transmigration of lymphocytes on human hepatic endothelial cells. *J Immunol*; 169: 983-92.
- LARSEN, P. J., FLEDELIUS, C., KNUDSEN, L. B. & TANG-CHRISTENSEN, M. 2001. Systemic administration of the long-acting GLP-1 derivative NN2211 induces lasting and reversible weight loss in both normal and obese rats. *Diabetes*; 50: 2530-2539.
- LEBOVITZ, H. E. & BANERJI, M. A. 2005. Point: visceral adiposity is causally related to insulin resistance. *Diabetes Care*; 28: 2322-5.
- LEE, J., HONG, S.-W., CHAE, S. W., KIM, D. H., CHOI, J. H., BAE, J. C., PARK, S. E., RHEE, E.-J., PARK, C.-Y., OH, K.-W., PARK, S.-W., KIM, S.-W. & LEE, W.-Y. 2012. Exendin-4 improves steatohepatitis by increasing Sirt1 expression in high-fat diet-induced obese C57BL/6J mice. *PLoS ONE*; 7: e31394.
- LEE, Y.-S., SHIN, S., SHIGIHARA, T., HAHM, E., LIU, M.-J., HAN, J., YOON, J.-W. & JUN, H.-S. 2007. Glucagon-like peptide-1 gene therapy in obese diabetic mice results in long-term cure

of diabetes by improving insulin sensitivity and reducing hepatic gluconeogenesis. *Diabetes*; 56: 1671-9.

LEPAGE, G. & ROY, C. C. 1986. Direct transesterification of all classes of lipids in a one-step reaction. *J Lipid Res*; 27: 114-20.

LEUSCHNER, U. F. H., LINDENTHAL, B., HERRMANN, G., ARNOLD, J. C., RÖSSLE, M., CORDES, H.-J., ZEUZEM, S., HEIN, J., BERG, T. & GROUP, N. S. 2010. High-dose ursodeoxycholic acid therapy for nonalcoholic steatohepatitis: a double-blind, randomized, placebo-controlled trial. *Hepatology*; 52: 472-9.

LEWIS, J. D., FERRARA, A., PENG, T., HEDDERSON, M., BILKER, W. B., QUESENBERRY, C. P., VAUGHN, D. J., NESSEL, L., SELBY, J. & STROM, B. L. 2011. Risk of bladder cancer among diabetic patients treated with pioglitazone: interim report of a longitudinal cohort study. *Diabetes Care*; 34: 916-22.

LI, Y., HANSOTIA, T., YUSTA, B., RIS, F., HALBAN, P. A. & DRUCKER, D. J. 2003. Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis. *J Biol Chem*; 278: 471-8.

LILFORD, R., BENTHAM, L., GIRLING, A., LITCHFIELD, I., LANCASHIRE, R., ARMSTRONG, D., JONES, R., MARTEAU, T., NEUBERGER, J., GILL, P., CRAMB, R., OLLIFF, S., ARNOLD, D., KHAN, K., ARMSTRONG, M., HOULIHAN, D., NEWSOME, P., CHILTON, P., MOONS, K. & ALTMAN, D. 2013a. Birmingham and Lambeth Liver Evaluation Testing Strategies (BALLETS): a prospective cohort study. *Health Technol Assess*; 17: 1-307.

LILFORD, R. J., BENTHAM, L. M., ARMSTRONG, M. J., NEUBERGER, J. & GIRLING, A. J. 2013b. What is the best strategy for investigating abnormal liver function tests in primary care? Implications from a prospective study. *BMJ Open*; 3: Online only.

LINCOFF, A. M., WOLSKI, K., NICHOLLS, S. J. & NISSEN, S. E. 2007. Pioglitazone and risk of cardiovascular events in patients with type 2 diabetes mellitus: a meta-analysis of randomized trials. *JAMA*; 298: 1180-8.



- LINDOR, K. D., KOWDLEY, K. V., HEATHCOTE, E. J., HARRISON, M. E., JORGENSEN, R., ANGULO, P., LYMP, J. F., BURGART, L. & COLIN, P. 2004. Ursodeoxycholic acid for treatment of nonalcoholic steatohepatitis: results of a randomized trial. *Hepatology*; 39: 770-778.
- LIU, Q., ADAMS, L., BROUDE, A., FERNANDEZ, R., BARON, A. D. & PARKES, D. G. 2010. The exenatide analogue AC3174 attenuates hypertension, insulin resistance, and renal dysfunction in Dahl salt-sensitive rats. *Cardiovasc Diabetol*; 9: 32.
- LOMONACO, R., ORTIZ-LOPEZ, C., ORSAK, B., FINCH, J., WEBB, A., BRIL, F., LOUDEN, C., TIO, F. & CUSI, K. 2011. Role of ethnicity in overweight and obese patients with nonalcoholic steatohepatitis. *Hepatology*; 54: 837-45.
- LOMONACO, R., ORTIZ-LOPEZ, C., ORSAK, B., WEBB, A., HARDIES, J., DARLAND, C., FINCH, J., GASTALDELLI, A., HARRISON, S., TIO, F. & CUSI, K. 2012. Effect of adipose tissue insulin resistance on metabolic parameters and liver histology in obese patients with nonalcoholic fatty liver disease. *Hepatology*; 55: 1389-97.
- LUDWIG, J., VIGGIANO, T. R., MCGILL, D. B. & OH, B. J. 1980. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc*; 55: 434-438.
- MACLUSKY, N. J., COOK, S., SCROCCHI, L., SHIN, J., KIM, J., VACCARINO, F., ASA, S. L. & DRUCKER, D. J. 2000. Neuroendocrine function and response to stress in mice with complete disruption of glucagon-like peptide-1 receptor signaling. *Endocrinology*; 141: 752-62.
- MADSBAD, S., SCHMITZ, O., RANSTAM, J., JAKOBSEN, G., MATTHEWS, D. R. & GROUP, N.-I. S. 2004. Improved glycemic control with no weight increase in patients with type 2 diabetes after once-daily treatment with the long-acting glucagon-like peptide 1 analog liraglutide (NN2211): a 12-week, double-blind, randomized, controlled trial. *Diabetes Care*; 27: 1335-42.
- MALAGUARNERA, M., GARGANTE, M. P., RUSSO, C., ANTIC, T., VACANTE, M., MALAGUARNERA, M., AVITABILE, T., LI VOLTI, G. & GALVANO, F. 2010. L-carnitine supplementation to diet: a new tool in treatment of nonalcoholic steatohepatitis--a randomized and controlled clinical trial. *Am J Gastroenterol*; 105: 1338-45.

- MALHI, H. & GORES, G. J. 2008. Cellular and molecular mechanisms of liver injury. *Gastroenterology*; 134: 1641-54.
- MANNUCCI, E. & ROTELLA, C. M. 2008. Future perspectives on glucagon-like peptide-1, diabetes and cardiovascular risk. *Nutr Metab Cardiovasc Dis*; 18: 639-45.
- MARCEAU, P., BIRON, S., HOULD, F. S., MARCEAU, S., SIMARD, S., THUNG, S. N. & KRAL, J. G. 1999. Liver pathology and the metabolic syndrome X in severe obesity. *J Clin Endocrinol Metab*; 84: 1513-7.
- MARCHESINI, G., BRIZI, M., BIANCHI, G., TOMASSETTI, S., BUGIANESI, E., LENZI, M., MCCULLOUGH, A. J., NATALE, S., FORLANI, G. & MELCHIONDA, N. 2001. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes*; 50: 1844-50.
- MARRE, M., SHAW, J., BRÄNDLE, M., BEBAKAR, W. M. W., KAMARUDDIN, N. A., STRAND, J., ZDRAVKOVIC, M., LE THI, T. D., COLAGIURI, S. & GROUP, L.-. 2009. Liraglutide, a once-daily human GLP-1 analogue, added to a sulphonylurea over 26 weeks produces greater improvements in glycaemic and weight control compared with adding rosiglitazone or placebo in subjects with Type 2 diabetes (LEAD-1 SU). *Diabet Med*; 26: 268-78.
- MASUOKA, H. C. & CHALASANI, N. 2013. Nonalcoholic fatty liver disease: an emerging threat to obese and diabetic individuals. *Ann N Y Acad Sci*; 1281: 106-22.
- MATTEONI, C. A., YOUNOSSI, Z. M., GRAMLICH, T., BOPARAI, N., LIU, Y. C. & MCCULLOUGH, A. J. 1999. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology*; 116: 1413-1419.
- MAYOR, S. 2010. NICE approves liraglutide for diabetic patients not achieving glucose control. *BMJ*; 341: c5062.
- MCGARRY, J. D. & BROWN, N. F. 1997. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur J Biochem*; 244: 1-14.

MCKIMMIE, R. L., DANIEL, K. R., CARR, J. J., BOWDEN, D. W., FREEDMAN, B. I., REGISTER, T. C., HSU, F.-C., LOHMAN, K. K., WEINBERG, R. B. & WAGENKNECHT, L. E. 2008. Hepatic steatosis and subclinical cardiovascular disease in a cohort enriched for type 2 diabetes: the Diabetes Heart Study. *Am J Gastroenterol*; 103: 3029-35.

MCLERNON, D. J., DONNAN, P. T., RYDER, S., RODERICK, P., SULLIVAN, F. M., ROSENBERG, W. & DILLON, J. F. 2009. Health outcomes following liver function testing in primary care: a retrospective cohort study. *Fam Pract*; 26: 251-9.

MCPHERSON, S., ANSTEE, Q. M., HENDERSON, E., DAY, C. P. & BURT, A. D. 2013. Are simple noninvasive scoring systems for fibrosis reliable in patients with NAFLD and normal ALT levels? *Eur J Gastroenterol Hepatol*; 25: 652-8.

MCPHERSON, S., JONSSON, J. R., COWIN, G. J., O'ROURKE, P., CLOUSTON, A. D., VOLP, A., HORSFALL, L., JOTHIMANI, D., FAWCETT, J., GALLOWAY, G. J., BENSON, M. & POWELL, E. E. 2009. Magnetic resonance imaging and spectroscopy accurately estimate the severity of steatosis provided the stage of fibrosis is considered. *J Hepatol*; 51: 389-97.

MCPHERSON, S., STEWART, S. F., HENDERSON, E., BURT, A. D. & DAY, C. P. 2010. Simple non-invasive fibrosis scoring systems can reliably exclude advanced fibrosis in patients with non-alcoholic fatty liver disease. *Gut*; 59: 1265-9.

MEIER, J. J. 2012. GLP-1 receptor agonists for individualized treatment of type 2 diabetes mellitus. *Nat Rev Endocrinol*; 8: 728-42.

MEIER, J. J., GALLWITZ, B., SALMEN, S., GOETZE, O., HOLST, J. J., SCHMIDT, W. E. & NAUCK, M. A. 2003. Normalization of glucose concentrations and deceleration of gastric emptying after solid meals during intravenous glucagon-like peptide 1 in patients with type 2 diabetes. *J Clin Endocrinol Metab*; 88: 2719-25.

MELLS, J. E., FU, P. P., SHARMA, S., OLSON, D., CHENG, L., HANDY, J. A., SAXENA, N. K., SORESCU, D. & ANANIA, F. A. 2012. Glp-1 analog, liraglutide, ameliorates hepatic steatosis and cardiac hypertrophy in C57BL/6J mice fed a Western diet. *Am J Physiol Gastrointest Liver Physiol*; 302: G225-35.

- MENEILLY, G. S., GREIG, N., TILDESLEY, H., HABENER, J. F., EGAN, J. M. & ELAHI, D. 2003. Effects of 3 months of continuous subcutaneous administration of glucagon-like peptide 1 in elderly patients with type 2 diabetes. *Diabetes Care*; 26: 2835-41.
- MENEILLY, G. S., MCINTOSH, C. H., PEDERSON, R. A., HABENER, J. F., GINGERICH, R., EGAN, J. M., FINEGOOD, D. T. & ELAHI, D. 2001. Effect of glucagon-like peptide 1 on non-insulin-mediated glucose uptake in the elderly patient with diabetes. *Diabetes Care*; 24: 1951-6.
- MILES, J. M. & JENSEN, M. D. 2005. Counterpoint: visceral adiposity is not causally related to insulin resistance. *Diabetes Care*; 28: 2326-8.
- MINERVINI, M. I., RUPPERT, K., FONTES, P., VOPES, R., VIZZINI, G., DE VERA, M. E., GRUTTADAURIA, S., MIRAGLIA, R., PIPITONE, L., MARSH, J. W., MARCOS, A., GRIDELLI, B. & DEMETRIS, A. J. 2009. Liver biopsy findings from healthy potential living liver donors: reasons for disqualification, silent diseases and correlation with liver injury tests. *J Hepatol*; 50: 501-10.
- MUMMADI, R. R., KASTURI, K. S., CHENNAREDDYGARI, S. & SOOD, G. K. 2008. Effect of bariatric surgery on nonalcoholic fatty liver disease: systematic review and meta-analysis. *Clin Gastroenterol Hepatol*; 6: 1396-402.
- MUSSO, G., CASSADER, M., DE MICHEL, F., ROSINA, F., ORLANDI, F. & GAMBINO, R. 2012. Nonalcoholic steatohepatitis versus steatosis: adipose tissue insulin resistance and dysfunctional response to fat ingestion predict liver injury and altered glucose and lipoprotein metabolism. *Hepatology*; 56: 933-42.
- MUSSO, G., GAMBINO, R., CASSADER, M. & PAGANO, G. 2010. A meta-analysis of randomized trials for the treatment of nonalcoholic fatty liver disease. *Hepatology*; 52: 79-104.
- MUSSO, G., GAMBINO, R., CASSADER, M. & PAGANO, G. 2011. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med*; 43: 617-49.

MYERS, R. P., POLLETT, A., KIRSCH, R., POMIER-LAYRARGUES, G., BEATON, M., LEVSTIK, M., DUARTE-ROJO, A., WONG, D., CROTTY, P. & ELKASHAB, M. 2012. Controlled Attenuation Parameter (CAP): a noninvasive method for the detection of hepatic steatosis based on transient elastography. *Liver Int*; 32: 902-10.

NACHNANI, J. S., BULCHANDANI, D. G., NOOKALA, A., HERNDON, B., MOLTENI, A., PANDYA, P., TAYLOR, R., QUINN, T., WEIDE, L. & ALBA, L. M. 2010. Biochemical and histological effects of exendin-4 (exenatide) on the rat pancreas. *Diabetologia*; 53: 153-9.

NADALIN, S., MALAGÓ, M., VALENTIN-GAMAZO, C., TESTA, G., BABA, H. A., LIU, C., FRÜHAUF, N. R., SCHAFFER, R., GERKEN, G., FRILLING, A. & BROELSCH, C. E. 2005. Preoperative donor liver biopsy for adult living donor liver transplantation: risks and benefits. *Liver Transpl*; 11: 980-6.

NAUCK, M., FRID, A., HERMANSEN, K., SHAH, N. S., TANKOVA, T., MITHA, I. H., ZDRAVKOVIC, M., DÜRING, M., MATTHEWS, D. R. & GROUP, L.-. 2009. Efficacy and safety comparison of liraglutide, glimepiride, and placebo, all in combination with metformin, in type 2 diabetes: the LEAD (liraglutide effect and action in diabetes)-2 study. *Diabetes Care*; 32: 84-90.

NAUCK, M., STÖCKMANN, F., EBERT, R. & CREUTZFELDT, W. 1986. Reduced incretin effect in type 2 (non-insulin-dependent) diabetes. *Diabetologia*; 29: 46-52.

NAUCK, M. A. 2013. A critical analysis of the clinical use of incretin-based therapies: The benefits by far outweigh the potential risks. *Diabetes Care*; 36: 2126-32.

NAUCK, M. A., DURAN, S., KIM, D., JOHNS, D., NORTHRUP, J., FESTA, A., BRODOWS, R. & TRAUTMANN, M. 2007. A comparison of twice-daily exenatide and biphasic insulin aspart in patients with type 2 diabetes who were suboptimally controlled with sulfonylurea and metformin: a non-inferiority study. *Diabetologia*; 50: 259-67.

NAUCK, M. A., HEIMESAAT, M. M., BEHLE, K., HOLST, J. J., NAUCK, M. S., RITZEL, R., HÜFNER, M. & SCHMIEGEL, W. H. 2002. Effects of glucagon-like peptide 1 on counterregulatory hormone responses, cognitive functions, and insulin secretion during hyperinsulinemic,

stepped hypoglycemic clamp experiments in healthy volunteers. *J Clin Endocrinol Metab*; 87: 1239-46.

NAUCK, M. A., HEIMESAAT, M. M., ORSKOV, C., HOLST, J. J., EBERT, R. & CREUTZFELDT, W. 1993. Preserved incretin activity of glucagon-like peptide 1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide in patients with type-2 diabetes mellitus. *J Clin Invest*; 91: 301-7.

NAUCK, M. A., NIEDEREICHHOLZ, U., ETTLER, R., HOLST, J. J., ORSKOV, C., RITZEL, R. & SCHMIEGEL, W. H. 1997. Glucagon-like peptide 1 inhibition of gastric emptying outweighs its insulinotropic effects in healthy humans. *Am J Physiol*; 273: E981-8.

NAUCK, M. A., VARDARLI, I., DEACON, C. F., HOLST, J. J. & MEIER, J. J. 2011. Secretion of glucagon-like peptide-1 (GLP-1) in type 2 diabetes: what is up, what is down? *Diabetologia*; 54: 10-8.

NCEP. 2002. Third Report of the NCEP Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation*; 106: 3143-3421.

NELSON, A., TORRES, D. M., MORGAN, A. E., FINCKE, C. & HARRISON, S. A. 2009. A pilot study using simvastatin in the treatment of nonalcoholic steatohepatitis: A randomized placebo-controlled trial. *J.Clin.Gastroenterol.*; 43: 990-994.

NEUSCHWANDER-TETRI, B. A., CLARK, J. M., BASS, N. M., VAN NATTA, M. L., UNALP-ARIDA, A., TONASCIA, J., ZEIN, C. O., BRUNT, E. M., KLEINER, D. E., MCCULLOUGH, A. J., SANYAL, A. J., DIEHL, A. M., LAVINE, J. E., CHALASANI, N., KOWDLEY, K. V. & NETWORK, N. C. R. 2010. Clinical, laboratory and histological associations in adults with nonalcoholic fatty liver disease. *Hepatology*; 52: 913-24.

NIELSEN, L. L., YOUNG, A. A. & PARKES, D. G. 2004a. Pharmacology of exenatide (synthetic exendin-4): a potential therapeutic for improved glycemic control of type 2 diabetes. *Regul Pept*; 117: 77-88.

- NIELSEN, S., GUO, Z., JOHNSON, C. M., HENSRUD, D. D. & JENSEN, M. D. 2004b. Splanchnic lipolysis in human obesity. *J Clin Invest*; 113: 1582-8.
- NIKOLAIDIS, L. A., ELAHI, D., SHEN, Y.-T. & SHANNON, R. P. 2005. Active metabolite of GLP-1 mediates myocardial glucose uptake and improves left ventricular performance in conscious dogs with dilated cardiomyopathy. *Am J Physiol Heart Circ Physiol*; 289: H2401-8.
- NIKOLAIDIS, L. A., MANKAD, S., SOKOS, G. G., MISKE, G., SHAH, A., ELAHI, D. & SHANNON, R. P. 2004. Effects of glucagon-like peptide-1 in patients with acute myocardial infarction and left ventricular dysfunction after successful reperfusion. *Circulation*; 109: 962-5.
- NOBILI, V., MANCO, M., DEVITO, R., DI, C. V., COMPARCOLA, D., SARTORELLI, M. R., PIEMONTE, F., MARCELLINI, M. & ANGULO, P. 2008. Lifestyle intervention and antioxidant therapy in children with nonalcoholic fatty liver disease: a randomized, controlled trial. *Hepatology*; 48: 119-128.
- NOEL, R. A., BRAUN, D. K., PATTERSON, R. E. & BLOOMGREN, G. L. 2009. Increased risk of acute pancreatitis and biliary disease observed in patients with type 2 diabetes: a retrospective cohort study. *Diabetes Care*; 32: 834-8.
- NORDESTGAARD, B. G., BENN, M., SCHNOHR, P. & TYBJAERG-HANSEN, A. 2007. Nonfasting triglycerides and risk of myocardial infarction, ischemic heart disease, and death in men and women. *JAMA*; 298: 299-308.
- NOVO NORDISK A/S. 2009. SmPc for Liraglutide (Victoza®).
- NOYAN-ASHRAF, M. H., SHIKATANI, E. A., SCHUIKI, I., MUKOVOZOV, I., WU, J., LI, R.-K., VOLCHUK, A., ROBINSON, L. A., BILLIA, F., DRUCKER, D. J. & HUSAIN, M. 2013. A glucagon-like peptide-1 analog reverses the molecular pathology and cardiac dysfunction of a mouse model of obesity. *Circulation*; 127: 74-85.
- NYBORG, N. C. B., MØLCK, A.-M., MADSEN, L. W. & KNUDSEN, L. B. 2012. The human GLP-1 analog liraglutide and the pancreas: evidence for the absence of structural pancreatic changes in three species. *Diabetes*; 61: 1243-9.

- OKERSON, T., YAN, P., STONEHOUSE, A. & BRODOWS, R. 2010. Effects of exenatide on systolic blood pressure in subjects with type 2 diabetes. *Am J Hypertens*; 23: 334-9.
- OLIVA, M. R., MORTELE, K. J., SEGATTO, E., GLICKMAN, J. N., ERTURK, S. M., ROS, P. R. & SILVERMAN, S. G. 2006. Computed tomography features of nonalcoholic steatohepatitis with histopathologic correlation. *J Comput Assist Tomogr*; 30: 37-43.
- ONG, J. P., ELARINY, H., COLLANTES, R., YOUNOSZAI, A., CHANDHOKE, V., REINES, H. D., GOODMAN, Z. & YOUNOSSI, Z. M. 2005. Predictors of nonalcoholic steatohepatitis and advanced fibrosis in morbidly obese patients. *Obes Surg*; 15: 310-5.
- ONO, M. & SAIBARA, T. 2012. Is impaired Kupffer cell function really important to the pathogenesis of nonalcoholic steatohepatitis? *J Gastroenterol Hepatol*; 27: 622-4.
- ORSKOV, C., HOLST, J. J. & NIELSEN, O. V. 1988. Effect of truncated glucagon-like peptide-1 [proglucagon-(78-107) amide] on endocrine secretion from pig pancreas, antrum, and nonantral stomach. *Endocrinology*; 123: 2009-13.
- ORSKOV, C., WETTERGREN, A. & HOLST, J. J. 1993. Biological effects and metabolic rates of glucagonlike peptide-1 7-36 amide and glucagonlike peptide-1 7-37 in healthy subjects are indistinguishable. *Diabetes*; 42: 658-61.
- ORSKOV, L., HOLST, J. J., MØLLER, J., ORSKOV, C., MØLLER, N., ALBERTI, K. G. & SCHMITZ, O. 1996. GLP-1 does not acutely affect insulin sensitivity in healthy man. *Diabetologia*; 39: 1227-32.
- ORTIZ-LOPEZ, C., LOMONACO, R., ORSAK, B., FINCH, J., CHANG, Z., KOCHUNOV, V. G., HARDIES, J. & CUSI, K. 2012. Prevalence of prediabetes and diabetes and metabolic profile of patients with nonalcoholic fatty liver disease (NAFLD). *Diabetes Care*; 35: 873-8.
- PANJWANI, N., MULVIHILL, E. E., LONGUET, C., YUSTA, B., CAMPBELL, J. E., BROWN, T. J., STREUTKER, C., HOLLAND, D., CAO, X., BAGGIO, L. L. & DRUCKER, D. J. 2013. GLP-1 receptor activation indirectly reduces hepatic lipid accumulation but does not attenuate development of atherosclerosis in diabetic male ApoE(-/-) mice. *Endocrinology*; 154: 127-39.



- PARK, S., HONG, S. M. & AHN, I. S. 2010. Exendin-4 and exercise improve hepatic glucose homeostasis by promoting insulin signaling in diabetic rats. *Metab Clin Exp*; 59: 123-33.
- PARK, S. H., KIM, P. N., KIM, K. W., LEE, S. W., YOON, S. E., PARK, S. W., HA, H. K., LEE, M.-G., HWANG, S., LEE, S.-G., YU, E. S. & CHO, E. Y. 2006. Macrovesicular hepatic steatosis in living liver donors: use of CT for quantitative and qualitative assessment. *Radiology*; 239: 105-12.
- PARKES, J., RODERICK, P., HARRIS, S., DAY, C., MUTIMER, D., COLLIER, J., LOMBARD, M., ALEXANDER, G., RAMAGE, J., DUSHEIKO, G., WHEATLEY, M., GOUGH, C., BURT, A. & ROSENBERG, W. 2010. Enhanced liver fibrosis test can predict clinical outcomes in patients with chronic liver disease. *Gut*; 59: 1245-51.
- PARKS, M. & ROSEBRAUGH, C. 2010. Weighing risks and benefits of liraglutide--the FDA's review of a new antidiabetic therapy. *N Engl J Med*; 362: 774-7.
- PARLEVLIET, E. T., SCHRÖDER-VAN DER ELST, J. P., CORSSMIT, E. P. M., PICHA, K., O'NEIL, K., STOJANOVIC-SUSULIC, V., ORT, T., HAVEKES, L. M., ROMIJN, J. A. & PIJL, H. 2009. CNT0736, a novel glucagon-like peptide-1 receptor agonist, ameliorates insulin resistance and inhibits very low-density lipoprotein production in high-fat-fed mice. *J Pharmacol Exp Ther*; 328: 240-8.
- PARLEVLIET, E. T., WANG, Y., GEERLING, J. J., SCHRÖDER-VAN DER ELST, J. P., PICHA, K., O'NEIL, K., STOJANOVIC-SUSULIC, V., ORT, T., HAVEKES, L. M., ROMIJN, J. A., PIJL, H. & RENSEN, P. C. N. 2012. GLP-1 receptor activation inhibits VLDL production and reverses hepatic steatosis by decreasing hepatic lipogenesis in high-fat-fed APOE\*3-Leiden mice. *PLoS ONE*; 7: e49152.
- PATEL, P. & ABATE, N. 2013. Role of subcutaneous adipose tissue in the pathogenesis of insulin resistance. *J Obes*; 2013: 489187.
- PEREA, A., VIÑAMBRES, C., CLEMENTE, F., VILLANUEVA-PEÑACARRILLO, M. L. & VALVERDE, I. 1997. GLP-1 (7-36) amide: effects on glucose transport and metabolism in rat adipose tissue. *Horm Metab Res*; 29: 417-21.

- PETERSEN, K. F., DUFOUR, S., FENG, J., BEFROY, D., DZIURA, J., DALLA MAN, C., COBELLI, C. & SHULMAN, G. I. 2006. Increased prevalence of insulin resistance and nonalcoholic fatty liver disease in Asian-Indian men. *Proc Natl Acad Sci USA*; 103: 18273-7.
- POLYZOS, S. A., KOUNTOURAS, J., ZAVOS, C. & TSIAOUI, E. 2010. The role of adiponectin in the pathogenesis and treatment of non-alcoholic fatty liver disease. *Diabetes Obes Metab*; 12: 365-83.
- POSTIC, C. & GIRARD, J. 2008. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J Clin Invest*; 118: 829-38.
- POYNARD, T., LASSAILLY, G., DIAZ, E., CLEMENT, K., CAIAZZO, R., TORDJMAN, J., MUNTEANU, M., PERAZZO, H., DEMOL, B., CALLAFE, R., PATTOU, F., CHARLOTTE, F., BEDOSSA, P., MATHURIN, P., RATZIU, V. & CONSORTIUM, F. 2012. Performance of biomarkers FibroTest, ActiTest, SteatoTest, and NashTest in patients with severe obesity: meta analysis of individual patient data. *PLoS ONE*; 7: e30325.
- POYNARD, T., LEBRAY, P., INGILIZ, P., VARAUT, A., VARSAT, B., NGO, Y., NORHA, P., MUNTEANU, M., DRANE, F., MESSOUS, D., BISMUT, F. I., CARRAU, J. P., MASSARD, J., RATZIU, V. & GIORDANELLA, J. P. 2010. Prevalence of liver fibrosis and risk factors in a general population using non-invasive biomarkers (FibroTest). *BMC Gastroenterol*; 10: 40.
- POYNARD, T., RATZIU, V., CHARLOTTE, F., MESSOUS, D., MUNTEANU, M., IMBERT-BISMUT, F., MASSARD, J., BONYHAY, L., TAHIRI, M., THABUT, D., CADRANEL, J. F., LE BAIL, B. & DE, L. V. 2006. Diagnostic value of biochemical markers (NashTest) for the prediction of non alcoholo steato hepatitis in patients with non-alcoholic fatty liver disease. *BMC Gastroenterol*; 6: 34.
- PRATI, D., COLLI, A., CONTE, D. & COLOMBO, M. 2005. Spectrum of NAFLD and diagnostic implications of the proposed new normal range for serum ALT in obese women. *Hepatology*; 42: 1460-1; author reply 1461.

PRATI, D., TAIOLI, E., ZANELLA, A., DELLA, T. E., BUTELLI, S., DEL VECCHIO, E., VIANELLO, L., ZANUSO, F., MOZZI, F., MILANI, S., CONTE, D., COLOMBO, M. & SIRCHIA, G. 2002. Updated definitions of healthy ranges for serum alanine aminotransferase levels. *Ann Intern Med*; 137: 1-10.

PRATLEY, R., NAUCK, M., BAILEY, T., MONTANYA, E., CUDDIHY, R., FILETTI, S., GARBER, A., THOMSEN, A. B., HARTVIG, H., DAVIES, M. & GROUP, -. L.-D.-. 2011. One year of liraglutide treatment offers sustained and more effective glycaemic control and weight reduction compared with sitagliptin, both in combination with metformin, in patients with type 2 diabetes: a randomised, parallel-group, open-label trial. *Int J Clin Pract*; 65: 397-407.

PRIGEON, R. L., QUDDUSI, S., PATY, B. & D'ALESSIO, D. A. 2003. Suppression of glucose production by GLP-1 independent of islet hormones: a novel extrapancreatic effect. *Am J Physiol Endocrinol Metab*; 285: E701-7.

PROMRAT, K., KLEINER, D. E., NIEMEIER, H. M., JACKVONY, E., KEARNS, M., WANDS, J. R., FAVA, J. L. & WING, R. R. 2010. Randomized controlled trial testing the effects of weight loss on nonalcoholic steatohepatitis. *Hepatology*; 51: 121-9.

PYKE, C. & KNUDSEN, L. B. 2013. The Glucagon-Like Peptide-1 Receptor--or Not? *Endocrinology*; 154: 4-8.

RAFIQ, N., BAI, C., FANG, Y., SRISHORD, M., MCCULLOUGH, A., GRAMLICH, T. & YOUNOSSI, Z. M. 2009. Long-term follow-up of patients with nonalcoholic fatty liver. *Clin Gastroenterol Hepatol*; 7: 234-8.

RATZIU, V., BELLENTANI, S., CORTEZ-PINTO, H., DAY, C. & MARCHESINI, G. 2010a. A position statement on NAFLD/NASH based on the EASL 2009 special conference. *J Hepatol*; 53: 372-84.

RATZIU, V., CHARLOTTE, F., BERNHARDT, C., GIRAL, P., HALBRON, M., LENAOUR, G., HARTMANN-HEURTIER, A., BRUCKERT, E., POYNARD, T. & GROUP, L. S. 2010b. Long-term efficacy of rosiglitazone in nonalcoholic steatohepatitis: results of the fatty liver improvement by rosiglitazone therapy (FLIRT 2) extension trial. *Hepatology*; 51: 445-53.

RATZIU, V., CHARLOTTE, F., HEURTIER, A., GOMBERT, S., GIRAL, P., BRUCKERT, E., GRIMALDI, A., CAPRON, F., POYNARD, T. & GROUP, L. S. 2005. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. *Gastroenterology*; 128: 1898-906.

RATZIU, V., GIRAL, P., JACQUEMINET, S., CHARLOTTE, F., HARTEMANN-HEURTIER, A., SERFATY, L., PODEVIN, P., LACORTE, J. M., BERNHARDT, C., BRUCKERT, E., GRIMALDI, A. & POYNARD, T. 2008. Rosiglitazone for nonalcoholic steatohepatitis: one-year results of the randomized placebo-controlled Fatty Liver Improvement with Rosiglitazone Therapy (FLIRT) Trial. *Gastroenterology*; 135: 100-110.

RATZIU, V., MASSARD, J., CHARLOTTE, F., MESSOUS, D., IMBERT-BISMUT, F., BONYHAY, L., TAHIRI, M., MUNTEANU, M., THABUT, D., CADRANEL, J. F., LE BAIL, B., DE LEDINGHEN, V., POYNARD, T., GROUP, L. S. & GROUP, C. S. 2006. Diagnostic value of biochemical markers (FibroTest-FibroSURE) for the prediction of liver fibrosis in patients with non-alcoholic fatty liver disease. *BMC Gastroenterol*; 6: 6.

READ, P. A., KHAN, F. Z. & DUTKA, D. P. 2012. Cardioprotection against ischaemia induced by dobutamine stress using glucagon-like peptide-1 in patients with coronary artery disease. *Heart*; 98: 408-13.

REDONDO, A., TRIGO, M. V., ACITORES, A., VALVERDE, I. & VILLANUEVA-PENACARRILLO, M. L. 2003. Cell signalling of the GLP-1 action in rat liver. *Mol Cell Endocrinol*; 204: 43-50.

REINER, T., THURBER, G., GAGLIA, J., VINEGONI, C., LIEW, C. W., UPADHYAY, R., KOHLER, R. H., LI, L., KULKARNI, R. N., BENOIST, C., MATHIS, D. & WEISSLEDER, R. 2011. Accurate measurement of pancreatic islet beta-cell mass using a second-generation fluorescent exendin-4 analog. *Proc Natl Acad Sci USA*; 108: 12815-20.

REINERT, D. F. & ALLEN, J. P. 2002. The Alcohol Use Disorders Identification Test (AUDIT): a review of recent research. *Alcohol Clin Exp Res*; 26: 272-279.

RICCHI, M., ODOARDI, M. R., CARULLI, L., ANZIVINO, C., BALLESTRI, S., PINETTI, A., FANTONI, L. I., MARRA, F., BERTOLOTTI, M., BANNI, S., LONARDO, A., CARULLI, N. & LORIA, P. 2009.

Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *J Gastroenterol Hepatol*; 24: 830-40.

RIDDLE, M. C., HENRY, R. R., POON, T. H., ZHANG, B., MAC, S. M., HOLCOMBE, J. H., KIM, D. D. & MAGGS, D. G. 2006. Exenatide elicits sustained glycaemic control and progressive reduction of body weight in patients with type 2 diabetes inadequately controlled by sulphonylureas with or without metformin. *Diabetes Metab Res Rev*; 22: 483-91.

ROBINSON, L. E., HOLT, T. A., REES, K., RANDEVA, H. S. & O'HARE, J. P. 2013. Effects of exenatide and liraglutide on heart rate, blood pressure and body weight: systematic review and meta-analysis. *BMJ Open*; 3: Online only.

ROCKEY, D. C. & BISSELL, D. M. 2006. Noninvasive measures of liver fibrosis. *Hepatology*; 43: S113-20.

ROLIN, B., LARSEN, M. O., GOTFREDSEN, C. F., DEACON, C. F., CARR, R. D., WILKEN, M. & KNUDSEN, L. B. 2002. The long-acting GLP-1 derivative NN2211 ameliorates glycemia and increases beta-cell mass in diabetic mice. *Am J Physiol Endocrinol Metab*; 283: E745-E752.

ROSENBERG, W. M. C., VOELKER, M., THIEL, R., BECKA, M., BURT, A., SCHUPPAN, D., HUBSCHER, S., ROSKAMS, T., PINZANI, M., ARTHUR, M. J. P. & GROUP, E. L. F. 2004. Serum markers detect the presence of liver fibrosis: a cohort study. *Gastroenterology*; 127: 1704-13.

RUIZ-GRANDE, C., ALARCÓN, C., MÉRIDA, E. & VALVERDE, I. 1992. Lipolytic action of glucagon-like peptides in isolated rat adipocytes. *Peptides*; 13: 13-6.

RUSSELL-JONES, D., CUDDIHY, R. M., HANEFELD, M., KUMAR, A., GONZÁLEZ, J. G., CHAN, M., WOLKA, A. M., BOARDMAN, M. K. & GROUP, D.-S. 2012. Efficacy and safety of exenatide once weekly versus metformin, pioglitazone, and sitagliptin used as monotherapy in drug-naive patients with type 2 diabetes (DURATION-4): a 26-week double-blind study. *Diabetes Care*; 35: 252-8.

RUSSELL-JONES, D. & GOUGH, S. 2012. Recent advances in incretin-based therapies. *Clin Endocrinol (Oxf)*; 77: 489-99.

RUSSELL-JONES, D., VAAG, A., SCHMITZ, O., SETHI, B. K., LALIC, N., ANTIC, S., ZDRAVKOVIC, M., RAVN, G. M., SIMÓ, R. & GROUP, L. 2009. Liraglutide vs insulin glargine and placebo in combination with metformin and sulfonylurea therapy in type 2 diabetes mellitus (LEAD-5 met+SU): a randomised controlled trial. *Diabetologia*; 52: 2046-55.

RYAN, A. S., EGAN, J. M., HABENER, J. F. & ELAHI, D. 1998. Insulinotropic hormone glucagon-like peptide-1-(7-37) appears not to augment insulin-mediated glucose uptake in young men during euglycemia. *J Clin Endocrinol Metab*; 83: 2399-404.

RYAN, C. K., JOHNSON, L. A., GERMIN, B. I. & MARCOS, A. 2002. One hundred consecutive hepatic biopsies in the workup of living donors for right lobe liver transplantation. *Liver Transpl*; 8: 1114-22.

SAADEH, S., YOUNOSSI, Z. M., REMER, E. M., GRAMLICH, T., ONG, J. P., HURLEY, M., MULLEN, K. D., COOPER, J. N. & SHERIDAN, M. J. 2002. The utility of radiological imaging in nonalcoholic fatty liver disease. *Gastroenterology*; 123: 745-50.

SAMSON, S. L. & BAJAJ, M. 2013. Direct actions of GLP-1 analogues on AMP-activated protein kinase activity are distinct from cyclic AMP accumulation. *J Hepatol*; 58: 634-5.

SAMSON, S. L., GONZALEZ, E. V., YECHOOR, V., BAJAJ, M., OKA, K. & CHAN, L. 2008. Gene therapy for diabetes: metabolic effects of helper-dependent adenoviral exendin 4 expression in a diet-induced obesity mouse model. *Mol Ther*; 16: 1805-1812.

SAMSON, S. L., SATHYANARAYANA, P., JOGI, M., GONZALEZ, E. V., GUTIERREZ, A., KRISHNAMURTHY, R., MUTHUPILLAI, R., CHAN, L. & BAJAJ, M. 2011. Exenatide decreases hepatic fibroblast growth factor 21 resistance in non-alcoholic fatty liver disease in a mouse model of obesity and in a randomised controlled trial. *Diabetologia*; 54: 3093-100.

SANCHO, V., TRIGO, M. V., GONZÁLEZ, N., VALVERDE, I., MALAISSE, W. J. & VILLANUEVA-PEÑACARRILLO, M. L. 2005. Effects of glucagon-like peptide-1 and exendins on kinase

activity, glucose transport and lipid metabolism in adipocytes from normal and type-2 diabetic rats. *J Mol Endocrinol*; 35: 27-38.

SANDHU, H., WIESENTHAL, S. R., MACDONALD, P. E., MCCALL, R. H., TCHIPASHVILI, V., RASHID, S., SATKUNARAJAH, M., IRWIN, D. M., SHI, Z. Q., BRUBAKER, P. L., WHEELER, M. B., VRANIC, M., EFENDIC, S. & GIACCA, A. 1999. Glucagon-like peptide 1 increases insulin sensitivity in depancreatized dogs. *Diabetes*; 48: 1045-53.

SANYAL, A. J., BANAS, C., SARGEANT, C., LUKETIC, V. A., STERLING, R. K., STRAVITZ, R. T., SHIFFMAN, M. L., HEUMAN, D., COTERRELL, A., FISHER, R. A., CONTOS, M. J. & MILLS, A. S. 2006. Similarities and differences in outcomes of cirrhosis due to nonalcoholic steatohepatitis and hepatitis C. *Hepatology*; 43: 682-9.

SANYAL, A. J., BRUNT, E. M., KLEINER, D. E., KOWDLEY, K. V., CHALASANI, N., LAVINE, J. E., RATZIU, V. & MCCULLOUGH, A. 2011. Endpoints and clinical trial design for nonalcoholic steatohepatitis. *Hepatology*; 54: 344-53.

SANYAL, A. J., CAMPBELL-SARGENT, C., MIRSHAHI, F., RIZZO, W. B., CONTOS, M. J., STERLING, R. K., LUKETIC, V. A., SHIFFMAN, M. L. & CLORE, J. N. 2001. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology*; 120: 1183-92.

SANYAL, A. J., CHALASANI, N., KOWDLEY, K. V., MCCULLOUGH, A., DIEHL, A. M., BASS, N. M., NEUSCHWANDER-TETRI, B. A., LAVINE, J. E., TONASCIA, J., UNALP, A., VAN NATTA, M., CLARK, J., BRUNT, E. M., KLEINER, D. E., HOOFNAGLE, J. H., ROBUCK, P. R. & CRN, N. 2010. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med*; 362: 1675-85.

SASSO, M., MIETTE, V., SANDRIN, L. & BEAUGRAND, M. 2012. The controlled attenuation parameter (CAP): a novel tool for the non-invasive evaluation of steatosis using Fibroscan. *Clin Res Hepatol Gastroenterol*; 36: 13-20.

- SCHIRRA, J., STURM, K., LEICHT, P., ARNOLD, R., GÖKE, B. & KATSCHINSKI, M. 1998. Exendin(9-39)amide is an antagonist of glucagon-like peptide-1(7-36)amide in humans. *J Clin Invest*; 101: 1421-30.
- SCHWIMMER, J. B., CELEDON, M. A., LAVINE, J. E., SALEM, R., CAMPBELL, N., SCHORK, N. J., SHIEHMORTEZA, M., YOKOO, T., CHAVEZ, A., MIDDLETON, M. S. & SIRLIN, C. B. 2009. Heritability of nonalcoholic fatty liver disease. *Gastroenterology*; 136: 1585-92.
- SCROCCHI, L. A., BROWN, T. J., MACLUSKY, N., BRUBAKER, P. L., AUERBACH, A. B., JOYNER, A. L. & DRUCKER, D. J. 1996. Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide 1 receptor gene. *Nat Med*; 2: 1254-8.
- SEGHIERI, M., REBELOS, E., GASTALDELLI, A., ASTIARRAGA, B. D., CASOLARO, A., BARSOTTI, E., POCAI, A., NAUCK, M., MUSCELLI, E. & FERRANNINI, E. 2013. Direct effect of GLP-1 infusion on endogenous glucose production in humans. *Diabetologia*; 56: 156-61.
- SHARMA, S., MELLS, J. E., FU, P. P., SAXENA, N. K. & ANANIA, F. A. 2011. GLP-1 Analogs Reduce Hepatocyte Steatosis and Improve Survival by Enhancing the Unfolded Protein Response and Promoting Macroautophagy. *PLoS ONE*; 6: e25269.
- SHAW, R. J., LAMIA, K. A., VASQUEZ, D., KOO, S.-H., BARDEESY, N., DEPINHO, R. A., MONTMINY, M. & CANTLEY, L. C. 2005. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science*; 310: 1642-6.
- SHEN, J., CHAN, H. L.-Y., WONG, G. L.-H., CHAN, A. W.-H., CHOI, P. C.-L., CHAN, H.-Y., CHIM, A. M.-L., YEUNG, D. K.-W., YU, J., CHU, W. C.-W. & WONG, V. W.-S. 2012. Assessment of non-alcoholic fatty liver disease using serum total cell death and apoptosis markers. *Aliment Pharmacol Ther*; 36: 1057-66.
- SHIELDS, W. W., THOMPSON, K. E., GRICE, G. A., HARRISON, S. A. & COYLE, W. J. 2009. The Effect of Metformin and Standard Therapy versus Standard Therapy alone in Nondiabetic Patients with Insulin Resistance and Nonalcoholic Steatohepatitis (NASH): A Pilot Trial. *Therap Adv Gastroenterol*; 2: 157-63.



- SHINDO, N., FUJISAWA, T., SUGIMOTO, K., NOJIMA, K., OZE-FUKAI, A., YOSHIKAWA, Y., WANG, X., YASUDA, O., IKEGAMI, H. & RAKUGI, H. 2010. Involvement of microsomal triglyceride transfer protein in nonalcoholic steatohepatitis in novel spontaneous mouse model. *J Hepatol*; 52: 903-12.
- SHIRAKAWA, J., FUJII, H., OHNUMA, K., SATO, K., ITO, Y., KAJI, M., SAKAMOTO, E., KOGANEI, M., SASAKI, H., NAGASHIMA, Y., AMO, K., AOKI, K., MORIMOTO, C., TAKEDA, E. & TERAUCHI, Y. 2011. Diet-induced adipose tissue inflammation and liver steatosis are prevented by DPP-4 inhibition in diabetic mice. *Diabetes*; 60: 1246-57.
- SINGH, S., CHANG, H.-Y., RICHARDS, T. M., WEINER, J. P., CLARK, J. M. & SEGAL, J. B. 2013. Glucagonlike peptide 1-based therapies and risk of hospitalization for acute pancreatitis in type 2 diabetes mellitus: a population-based matched case-control study. *JAMA*; 173: 534-9.
- SKELLY, M. M., JAMES, P. D. & RYDER, S. D. 2001. Findings on liver biopsy to investigate abnormal liver function tests in the absence of diagnostic serology. *J Hepatol*; 35: 195-9.
- SÖDERBERG, C., STÅL, P., ASKLING, J., GLAUMANN, H., LINDBERG, G., MARMUR, J. & HULTCRANTZ, R. 2010. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. *Hepatology*; 51: 595-602.
- SOKOS, G. G., BOLUKOGLU, H., GERMAN, J., HENTOSZ, T., MAGOVERN, G. J., MAHER, T. D., DEAN, D. A., BAILEY, S. H., MARRONE, G., BENCKART, D. H., ELAHI, D. & SHANNON, R. P. 2007. Effect of glucagon-like peptide-1 (GLP-1) on glycemic control and left ventricular function in patients undergoing coronary artery bypass grafting. *Am J Cardiol*; 100: 824-9.
- STEELE, R. 1959. Use of C14-glucose to measure hepatic glucose production following an intravenous glucose load or after injection of insulin. *Metab Clin Exp*; 8: 512-9.
- STURIS, J., GOTFREDSEN, C. F., RØMER, J., ROLIN, B., RIBEL, U., BRAND, C. L., WILKEN, M., WASSERMANN, K., DEACON, C. F., CARR, R. D. & KNUDSEN, L. B. 2003. GLP-1 derivative liraglutide in rats with beta-cell deficiencies: influence of metabolic state on beta-cell mass dynamics. *Br J Pharmacol*; 140: 123-32.

- SUZUKI, A., ANGULO, P., LYMP, J., ST SAUVER, J., MUTO, A., OKADA, T. & LINDOR, K. 2005. Chronological development of elevated aminotransferases in a nonalcoholic population. *Hepatology*; 41: 64-71.
- SUZUKI, A., LYMP, J., SAUVER, J. S., ANGULO, P. & LINDOR, K. 2006. Values and limitations of serum aminotransferases in clinical trials of nonalcoholic steatohepatitis. *Liver Int*; 26: 1209-16.
- SVEGLIATI-BARONI, G., SACCOMANNO, S., RYCHLICKI, C., AGOSTINELLI, L., DE MINICIS, S., CANDELAESI, C., FARACI, G., PACETTI, D., VIVARELLI, M., NICOLINI, D., GARELLI, P., CASINI, A., MANCO, M., MINGRONE, G., RISALITI, A., FREGA, G. N., BENEDETTI, A. & GASTALDELLI, A. 2011. Glucagon-like peptide-1 receptor activation stimulates hepatic lipid oxidation and restores hepatic signalling alteration induced by a high-fat diet in nonalcoholic steatohepatitis. *Liver Int*; 31: 1285-97.
- TALWALKAR, J. A., KURTZ, D. M., SCHOENLEBER, S. J., WEST, C. P. & MONTORI, V. M. 2007. Ultrasound-based transient elastography for the detection of hepatic fibrosis: systematic review and meta-analysis. *Clin Gastroenterol Hepatol*; 5: 1214-20.
- TAMURA, S. & SHIMOMURA, I. 2005. Contribution of adipose tissue and de novo lipogenesis to nonalcoholic fatty liver disease. *J Clin Invest*; 115: 1139-42.
- TARGHER, G., BERTOLINI, L., PADOVANI, R., RODELLA, S., TESSARI, R., ZENARI, L., DAY, C. & ARCARO, G. 2007. Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. *Diabetes Care*; 30: 1212-8.
- TETRI, L. H., BASARANOGLU, M., BRUNT, E. M., YERIAN, L. M. & NEUSCHWANDER-TETRI, B. A. 2008. Severe NAFLD with hepatic necroinflammatory changes in mice fed trans fats and a high-fructose corn syrup equivalent. *Am J Physiol Gastrointest Liver Physiol*; 295: G987-G995.
- THOMA, C., DAY, C. P. & TRENELL, M. I. 2012. Lifestyle interventions for the treatment of non-alcoholic fatty liver disease in adults: a systematic review. *J Hepatol*; 56: 255-66.

- THORENS, B. 1992. Expression cloning of the pancreatic beta cell receptor for the glucoregulatory hormone glucagon-like peptide 1. *Proc Natl Acad Sci USA*; 89: 8641-5.
- THORENS, B., PORRET, A., BÜHLER, L., DENG, S. P., MOREL, P. & WIDMANN, C. 1993. Cloning and functional expression of the human islet GLP-1 receptor. Demonstration that exendin-4 is an agonist and exendin-(9-39) an antagonist of the receptor. *Diabetes*; 42: 1678-82.
- TIMLIN, M. T. & PARKS, E. J. 2005. Temporal pattern of de novo lipogenesis in the postprandial state in healthy men. *Am J Clin Nutr*; 81: 35-42.
- TOFT-NIELSON, M., MADSBAD, S. & HOLST, J. J. 1996. The effect of glucagon-like peptide I (GLP-I) on glucose elimination in healthy subjects depends on the pancreatic glucoregulatory hormones. *Diabetes*; 45: 552-6.
- TOMAS, E. & HABENER, J. F. 2010. Insulin-like actions of glucagon-like peptide-1: a dual receptor hypothesis. *Trends Endocrinol Metab*; 21: 59-67.
- TOMAS, E., WOOD, J. A., STANOJEVIC, V. & HABENER, J. F. 2011a. GLP-1-derived nonapeptide GLP-1(28-36)amide inhibits weight gain and attenuates diabetes and hepatic steatosis in diet-induced obese mice. *Regul Pept*; 169: 43-8.
- TOMAS, E., WOOD, J. A., STANOJEVIC, V. & HABENER, J. F. 2011b. Glucagon-like peptide-1(9-36)amide metabolite inhibits weight gain and attenuates diabetes and hepatic steatosis in diet-induced obese mice. *Diabetes Obes Metab*; 13: 26-33.
- TORDJMAN, J., DIVOUX, A., PRIFTI, E., POITOU, C., PELLOUX, V., HUGOL, D., BASDEVANT, A., BOUILLOT, J.-L., CHEVALLIER, J.-M., BEDOSSA, P., GUERRE-MILLO, M. & CLEMENT, K. 2012. Structural and inflammatory heterogeneity in subcutaneous adipose tissue: relation with liver histopathology in morbid obesity. *J Hepatol*; 56: 1152-8.
- TORDJMAN, J., POITOU, C., HUGOL, D., BOUILLOT, J.-L., BASDEVANT, A., BEDOSSA, P., GUERRE-MILLO, M. & CLEMENT, K. 2009. Association between omental adipose tissue macrophages and liver histopathology in morbid obesity: influence of glycemic status. *J Hepatol*; 51: 354-62.

- TORRES, D. M., JONES, F. J., SHAW, J. C., WILLIAMS, C. D., WARD, J. A. & HARRISON, S. A. 2011. Rosiglitazone versus rosiglitazone and metformin versus rosiglitazone and losartan in the treatment of nonalcoholic steatohepatitis in humans: a 12-month randomized, prospective, open-label trial. *Hepatology*; 54: 1631-9.
- TRAN, T. T., CHANGSRI, C., SHACKLETON, C. R., POORDAD, F. F., NISSEN, N. N., COLQUHOUN, S., GELLER, S. A., VIERLING, J. M. & MARTIN, P. 2006. Living donor liver transplantation: histological abnormalities found on liver biopsies of apparently healthy potential donors. *J Gastroenterol Hepatol*; 21: 381-3.
- TREVASKIS, J. L., GRIFFIN, P. S., WITTMER, C., NEUSCHWANDER-TETRI, B. A., BRUNT, E. M., DOLMAN, C. S., ERICKSON, M. R., NAPORA, J., PARKES, D. G. & ROTH, J. D. 2012. Glucagon-like peptide-1 receptor agonism improves metabolic, biochemical, and histopathological indices of nonalcoholic steatohepatitis in mice. *Am J Physiol Gastrointest Liver Physiol*; 302: G762-72.
- TSOCHATZIS, E. A., GURUSAMY, K. S., NTAOULA, S., CHOLONGITAS, E., DAVIDSON, B. R. & BURROUGHS, A. K. 2011. Elastography for the diagnosis of severity of fibrosis in chronic liver disease: a meta-analysis of diagnostic accuracy. *J Hepatol*; 54: 650-9.
- TURTON, M. D., O'SHEA, D., GUNN, I., BEAK, S. A., EDWARDS, C. M., MEERAN, K., CHOI, S. J., TAYLOR, G. M., HEATH, M. M., LAMBERT, P. D., WILDING, J. P., SMITH, D. M., GHATEI, M. A., HERBERT, J. & BLOOM, S. R. 1996. A role for glucagon-like peptide-1 in the central regulation of feeding. *Nature*; 379: 69-72.
- TUSHUIZEN, M. E., BUNCK, M. C., POUWELS, P. J., VAN WAESBERGHE, J. H. T., DIAMANT, M. & HEINE, R. J. 2006. Incretin mimetics as a novel therapeutic option for hepatic steatosis. *Liver Int*; 26: 1015-7.
- UNGER, J. R. & PARKIN, C. G. 2011. Glucagon-like peptide-1 (GLP-1) receptor agonists: Differentiating the new medications. *Diabetes Ther*; 2: 29-39.

UNGER, R. H., OHNEDA, A., VALVERDE, I., EISENTRAUT, A. M. & EXTON, J. 1968. Characterization of the responses of circulating glucagon-like immunoreactivity to intraduodenal and intravenous administration of glucose. *J Clin Invest*; 47: 48-65.

US Food and Drug Administration, F. 2013. Victoza approval package. FDA/Centre for Drug Evaluation and Research. Available at:

[http://www.accessdata.fda.gov/drugsatfda\\_docs/nda/2010/022341s000TOC.cfm](http://www.accessdata.fda.gov/drugsatfda_docs/nda/2010/022341s000TOC.cfm) [Accessed on 1/09/2013].

UYGUN, A., KADAYIFCI, A., ISIK, A. T., OZGURTAS, T., DEVECI, S., TUZUN, A., YESILOVA, Z., GULSEN, M. & DAGALP, K. 2004. Metformin in the treatment of patients with non-alcoholic steatohepatitis. *Aliment Pharmacol Ther*; 19: 537-44.

VALLET-PICHARD, A., MALLET, V., NALPAS, B., VERKARRE, V., NALPAS, A., DHALLUIN-VENIER, V., FONTAINE, H. & POL, S. 2007. FIB-4: an inexpensive and accurate marker of fibrosis in HCV infection. comparison with liver biopsy and fibrotest. *Hepatology*; 46: 32-6.

VALLET-PICHARD, A., MALLET, V. & POL, S. 2006. FIB-4: a simple, inexpensive and accurate marker of fibrosis in HCV-infected patients. *Hepatology*; 44: 769; author reply 769-70.

VALVERDE, I., MORALES, M., CLEMENTE, F., LÓPEZ-DELGADO, M. I., DELGADO, E., PEREA, A. & VILLANUEVA-PEÑACARRILLO, M. L. 1994. Glucagon-like peptide 1: a potent glycogenic hormone. *FEBS Lett*; 349: 313-6.

VAN DER POORTEN, D., MILNER, K.-L., HUI, J., HODGE, A., TRENELL, M. I., KENCH, J. G., LONDON, R., PEDUTO, T., CHISHOLM, D. J. & GEORGE, J. 2008. Visceral fat: a key mediator of steatohepatitis in metabolic liver disease. *Hepatology*; 48: 449-57.

VAN WAGNER, L. B., KOPPE, S. W. P., BRUNT, E. M., GOTTSTEIN, J., GARDIKIOTES, K., GREEN, R. M. & RINELLA, M. E. 2011. Pentoxifylline for the treatment of non-alcoholic steatohepatitis: a randomized controlled trial. *Ann Hepatol*; 10: 277-86.

- VANNI, E., BUGIANESI, E., KOTRONEN, A., DE MINICIS, S., YKI-JÄRVINEN, H. & SVEGLIATI-BARONI, G. 2010. From the metabolic syndrome to NAFLD or vice versa? *Dig Liver Dis*; 42: 320-30.
- VELLA, A., SHAH, P., REED, A. S., ADKINS, A. S., BASU, R. & RIZZA, R. A. 2002. Lack of effect of exendin-4 and glucagon-like peptide-1-(7,36)-amide on insulin action in non-diabetic humans. *Diabetologia*; 45: 1410-5.
- VENDRELL, J., EL BEKAY, R., PERAL, B., GARCÍA-FUENTES, E., MEGIA, A., MACIAS-GONZALEZ, M., FERNÁNDEZ REAL, J., JIMENEZ-GOMEZ, Y., ESCOTÉ, X., PACHÓN, G., SIMÓ, R., SELVA, D. M., MALAGÓN, M. M. & TINAHONES, F. J. 2011. Study of the potential association of adipose tissue GLP-1 receptor with obesity and insulin resistance. *Endocrinology*; 152: 4072-9.
- VERSPÖHL, E. J. 2009. Novel therapeutics for type 2 diabetes: incretin hormone mimetics (glucagon-like peptide-1 receptor agonists) and dipeptidyl peptidase-4 inhibitors. *Pharmacol Ther*; 124: 113-38.
- VILLANUEVA-PEÑACARRILLO, M. L., MÁRQUEZ, L., GONZÁLEZ, N., DÍAZ-MIGUEL, M. & VALVERDE, I. 2001. Effect of GLP-1 on lipid metabolism in human adipocytes. *Horm Metab Res*; 33: 73-7.
- VILSBØLL, T. 2009. The effects of glucagon-like peptide-1 on the beta cell. *Diabetes Obes Metab*; 11 Suppl 3: 11-8.
- VRANG, N., JELSING, J., SIMONSEN, L., JENSEN, A. E., THORUP, I., SØEBORG, H. & KNUDSEN, L. B. 2012. The effects of 13 wk of liraglutide treatment on endocrine and exocrine pancreas in male and female ZDF rats: a quantitative and qualitative analysis revealing no evidence of drug-induced pancreatitis. *Am J Physiol Endocrinol Metab*; 303: E253-64.
- VUPPALANCHI, R. & CHALASANI, N. 2009. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: Selected practical issues in their evaluation and management. *Hepatology*; 49: 306-17.

- WAI, C.-T., GREENSON, J. K., FONTANA, R. J., KALBFLEISCH, J. D., MARRERO, J. A., CONJEEVARAM, H. S. & LOK, A. S.-F. 2003. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology*; 38: 518-26.
- WAKIL, S. J., STOOPS, J. K. & JOSHI, V. C. 1983. Fatty acid synthesis and its regulation. *Annu Rev Biochem*; 52: 537-79.
- WALLACE, T. M., LEVY, J. C. & MATTHEWS, D. R. 2004. Use and abuse of HOMA modeling. *Diabetes Care*; 27: 1487-95.
- WANG, B., ZHONG, J., LIN, H., ZHAO, Z., YAN, Z., HE, H., NI, Y., LIU, D. & ZHU, Z. 2013a. Blood pressure-lowering effects of GLP-1 receptor agonists exenatide and liraglutide: a meta-analysis of clinical trials. *Diabetes Obes Metab*; 15: 737-49.
- WANG, P., GUO, Q.-S., WANG, Z.-W. & QIAN, H.-X. 2013b. HBx induces HepG-2 cells autophagy through PI3K/Akt-mTOR pathway. *Mol Cell Biochem*; 372: 161-8.
- WANG, Y., KOLE, H. K., MONTROSE-RAFIZADEH, C., PERFETTI, R., BERNIER, M. & EGAN, J. M. 1997. Regulation of glucose transporters and hexose uptake in 3T3-L1 adipocytes: glucagon-like peptide-1 and insulin interactions. *J Mol Endocrinol*; 19: 241-8.
- WARE, J. E. 2008. Improvements in short-form measures of health status: introduction to a series. *J Clin Epidemiol*; 61: 1-5.
- WEI, Y. & MOJSOV, S. 1995. Tissue-specific expression of the human receptor for glucagon-like peptide-I: brain, heart and pancreatic forms have the same deduced amino acid sequences. *FEBS Lett*; 358: 219-24.
- WHALLEY, S., PUVANACHANDRA, P., DESAI, A. & KENNEDY, H. 2007. Hepatology outpatient service provision in secondary care: a study of liver disease incidence and resource costs. *Clin Med*; 7: 119-24.
- WILLIAMS, A. L. & HOOFNAGLE, J. H. 1988. Ratio of serum aspartate to alanine aminotransferase in chronic hepatitis. Relationship to cirrhosis. *Gastroenterology*; 95: 734-9.

WILLIAMS, C. D., STENGEL, J., ASIKE, M. I., TORRES, D. M., SHAW, J., CONTRERAS, M., LANDT, C. L. & HARRISON, S. A. 2011. Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterology*; 140: 124-31.

WILLIAMS, D. L., BASKIN, D. G. & SCHWARTZ, M. W. 2006. Leptin regulation of the anorexic response to glucagon-like peptide-1 receptor stimulation. *Diabetes*; 55: 3387-93.

WILLIAMSON, R. M., PRICE, J. F., GLANCY, S., PERRY, E., NEE, L. D., HAYES, P. C., FRIER, B. M., VAN LOOK, L. A. F., JOHNSTON, G. I., REYNOLDS, R. M., STRACHAN, M. W. J. & INVESTIGATORS, E. 2011. Prevalence of and risk factors for hepatic steatosis and nonalcoholic Fatty liver disease in people with type 2 diabetes: the Edinburgh Type 2 Diabetes Study. *Diabetes Care*; 34: 1139-44.

WONG, V. W.-S., CHU, W. C.-W., WONG, G. L.-H., CHAN, R. S.-M., CHIM, A. M.-L., ONG, A., YEUNG, D. K.-W., YIU, K. K.-L., CHU, S. H.-T., WOO, J., CHAN, F. K.-L. & CHAN, H. L.-Y. 2012a. Prevalence of non-alcoholic fatty liver disease and advanced fibrosis in Hong Kong Chinese: a population study using proton-magnetic resonance spectroscopy and transient elastography. *Gut*; 61: 409-15.

WONG, V. W.-S., VERGNIOL, J., WONG, G. L.-H., FOUCHER, J., CHAN, A. W.-H., CHERMAK, F., CHOI, P. C.-L., MERROUCHE, W., CHU, S. H.-T., PESQUE, S., CHAN, H. L.-Y. & DE LÉDINGHEN, V. 2012b. Liver stiffness measurement using XL probe in patients with nonalcoholic fatty liver disease. *Am J Gastroenterol*; 107: 1862-71.

WONG, V. W.-S., VERGNIOL, J., WONG, G. L.-H., FOUCHER, J., CHAN, H. L.-Y., LE BAIL, B., CHOI, P. C.-L., KOWO, M., CHAN, A. W.-H., MERROUCHE, W., SUNG, J. J.-Y. & DE LÉDINGHEN, V. 2010a. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology*; 51: 454-62.

WONG, V. W.-S., WONG, G. L.-H., CHIM, A. M.-L., TSE, A. M.-L., TSANG, S. W.-C., HUI, A. Y., CHOI, P. C.-L., CHAN, A. W.-H., SO, W.-Y., CHAN, F. K.-L., SUNG, J. J.-Y. & CHAN, H. L.-Y. 2008.



Validation of the NAFLD fibrosis score in a Chinese population with low prevalence of advanced fibrosis. *Am J Gastroenterol*; 103: 1682-8.

WONG, V. W.-S., WONG, G. L.-H., CHOI, P. C.-L., CHAN, A. W.-H., LI, M. K.-P., CHAN, H.-Y., CHIM, A. M.-L., YU, J., SUNG, J. J.-Y. & CHAN, H. L.-Y. 2010b. Disease progression of non-alcoholic fatty liver disease: a prospective study with paired liver biopsies at 3 years. *Gut*; 59: 969-74.

Thermo Scientific. Available at:

[http://www.thermo.com/eThermo/CMA/PDFs/Product/productPDF\\_27059.pdf](http://www.thermo.com/eThermo/CMA/PDFs/Product/productPDF_27059.pdf) [Accessed on 1/09/2013].

Thermo Scientific. Available at:

[http://www.thermo.com/eThermo/CMA/PDFs/Product/productPDF\\_27060.pdf](http://www.thermo.com/eThermo/CMA/PDFs/Product/productPDF_27060.pdf) [Accessed on 1/09/2013].

XU, A., WANG, Y., KESHAW, H., XU, L. Y., LAM, K. S. L. & COOPER, G. J. S. 2003. The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. *J Clin Invest*; 112: 91-100.

YAMADA, C., YAMADA, Y., TSUKIYAMA, K., YAMADA, K., UDAGAWA, N., TAKAHASHI, N., TANAKA, K., DRUCKER, D. J., SEINO, Y. & INAGAKI, N. 2008. The murine glucagon-like peptide-1 receptor is essential for control of bone resorption. *Endocrinology*; 149: 574-9.

YAMAMOTO, K., TAKADA, Y., FUJIMOTO, Y., HAGA, H., OIKE, F., KOBAYASHI, N. & TANAKA, K. 2007. Nonalcoholic steatohepatitis in donors for living donor liver transplantation. *Transplantation*; 83: 257-62.

YANG, H., EGAN, J. M., WANG, Y., MOYES, C. D., ROTH, J., MONTROSE, M. H. & MONTROSE-RAFIZADEH, C. 1998. GLP-1 action in L6 myotubes is via a receptor different from the pancreatic GLP-1 receptor. *Am J Physiol*; 275: C675-83.

- YE, J., COULOURIS, G., ZARETSKAYA, I., CUTCUTACHE, I., ROZEN, S. & MADDEN, T. L. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*; 13: 134.
- YONEDA, M., SUZUKI, K., KATO, S., FUJITA, K., NOZAKI, Y., HOSONO, K., SAITO, S. & NAKAJIMA, A. 2010. Nonalcoholic fatty liver disease: US-based acoustic radiation force impulse elastography. *Radiology*; 256: 640-7.
- YOUNG, A. A., GEDULIN, B. R., BHAVSAR, S., BODKIN, N., JODKA, C., HANSEN, B. & DENARO, M. 1999. Glucose-lowering and insulin-sensitizing actions of exendin-4: studies in obese diabetic (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (*Macaca mulatta*). *Diabetes*; 48: 1026-34.
- YUAN, M., KONSTANTOPOULOS, N., LEE, J., HANSEN, L., LI, Z. W., KARIN, M. & SHOELSON, S. E. 2001. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of *Ikkbeta*. *Science*; 293: 1673-7.
- ZANDER, M., MADSBAD, S., MADSEN, J. L. & HOLST, J. J. 2002. Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. *Lancet*; 359: 824-30.
- ZEIN, C. O., YERIAN, L. M., GOGATE, P., LOPEZ, R., KIRWAN, J. P., FELDSTEIN, A. E. & MCCULLOUGH, A. J. 2011. Pentoxifylline improves nonalcoholic steatohepatitis: a randomized placebo-controlled trial. *Hepatology*; 54: 1610-9.
- ZELBER-SAGI, S., KESSLER, A., BRAZOWSKY, E., WEBB, M., LURIE, Y., SANTO, M., LESHNO, M., BLENDIS, L., HALPERN, Z. & OREN, R. 2006. A double-blind randomized placebo-controlled trial of orlistat for the treatment of nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol*; 4: 639-44.
- ZHANG, L., YANG, M., REN, H., HU, H., BODEN, G., LI, L. & YANG, G. 2013. GLP-1 analogue prevents NAFLD in ApoE KO mice with diet and Acrp30 knockdown by inhibiting c-JNK. *Liver Int*; 33: 794-804.

ZHANG, X., XU, A., CHUNG, S. K., CRESSER, J. H. B., SWEENEY, G., WONG, R. L. C., LIN, A. & LAM, K. S. L. 2011. Selective inactivation of c-Jun NH2-terminal kinase in adipose tissue protects against diet-induced obesity and improves insulin sensitivity in both liver and skeletal muscle in mice. *Diabetes*; 60: 486-95.

ZHENG, D., IONUT, V., MOORADIAN, V., STEFANOVSKI, D. & BERGMAN, R. N. 2009. Exenatide sensitizes insulin-mediated whole-body glucose disposal and promotes uptake of exogenous glucose by the liver. *Diabetes*; 58: 352-9.

ZINMAN, B., GERICH, J., BUSE, J. B., LEWIN, A., SCHWARTZ, S., RASKIN, P., HALE, P. M., ZDRAVKOVIC, M., BLONDE, L. & INVESTIGATORS, L.-. 2009. Efficacy and safety of the human glucagon-like peptide-1 analog liraglutide in combination with metformin and thiazolidinedione in patients with type 2 diabetes (LEAD-4 Met+TZD). *Diabetes Care*; 32: 1224-30.

ZINMAN, B., HOOGWERF, B. J., DURÁN GARCÍA, S., MILTON, D. R., GIACONIA, J. M., KIM, D. D., TRAUTMANN, M. E. & BRODOWS, R. G. 2007. The effect of adding exenatide to a thiazolidinedione in suboptimally controlled type 2 diabetes: a randomized trial. *Ann Intern Med*; 146: 477-85.

## APPENDIX I

List of peer-reviewed publications by Armstrong MJ and colleagues that are directly related to the studies performed in the PhD:

### Original articles:

**Armstrong MJ**, Hazlehurst JM, Hull D, et al. Abdominal subcutaneous adipose tissue insulin resistance and lipolysis in patients with non-alcoholic steatohepatitis. *Diabetes Obes Metab.* 2014 (*accepted in press*).

**Armstrong MJ**, Barton D, Gaunt P, et al; LEAN trial team. Liraglutide efficacy and action in non-alcoholic steatohepatitis (LEAN): study protocol for a phase II multicentre, double-blinded, randomised, controlled trial. *BMJ Open.* 2013;3(11):e003995.

**Armstrong MJ**, Houlihan DD, Rowe IA, Clausen WH, Elbrønd B, Gough SC, Tomlinson JW, Newsome PN. Safety and efficacy of liraglutide in patients with type 2 diabetes and elevated liver enzymes: individual patient data meta-analysis of the LEAD program. *Aliment Pharmacol Ther.* 2013;37:234-42.

**Armstrong MJ**, Houlihan DD, Bentham L, Shaw JC, Cramb R, Olliff S, Gill PS, Neuberger JM, Lilford RJ, Newsome PN. Presence and severity of nonalcoholic liver disease in a large prospective primary care cohort. *J Hepatol.* 2012;56:234-40.

### Review articles:

**Armstrong MJ**, Adams LA, Canbay A, Syn WK. Extra-hepatic complications of nonalcoholic fatty liver disease. *Hepatology.* 2014;59:1174-97.

Dowman JK, **Armstrong MJ**, Tomlinson JW, Newsome PN. Current therapeutic strategies in non-alcoholic fatty liver disease. *Diabetes Obes Metab*. 2011; 13:692-702.

Letters:

**Armstrong MJ**, Houlihan DD, Rowe IA. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med*. 2010 Sep 16; 363(12):1185.